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Epstein-Barr Virus–induced Autoimmune Responses

II. Immunoglobulin G Autoantibodies to Mimicking and Nonmimicking Epitopes. Presence in Autoimmune Disease

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Abstract

During infectious mononucleosis, IgM autoantibodies are generated to a protein, p542, which contains a glycine-rich 28-mer epitope cross-reactive with the Epstein-Barr nuclear antigen-1 through Epstein-Barr nuclear antigen-1's glycine/alanine repeat. In normal individuals it is uncommon to find IgG anti-p542, but among patients with progressive systemic sclerosis, systemic lupus erythematosus, and ulcerative colitis high IgG anti-p542 (> 3 SD above the mean of normal 20–50 yr controls) occurred frequently. Lesser elevations occurred in Sjögren's syndrome, rheumatoid arthritis, ankylosing spondylitis, and Crohn's disease, but none with chronic hepatitis B infection. The reactive epitopes on p542 were mapped with deletion mutants, which indicated that the glycine-rich 28-mer was the major antigenic determinant, with lesser antibody responses to other epitopes. We conclude that normally there is an inability to generate IgG autoantibodies to the cross-reactive (mimicking) epitope of the p542 host protein, but that this inability is overcome in a proportion of patients with autoimmune disease. We conclude also that non–cross-reactive autoepitopes exist on p542 protein, to which IgG autoantibodies can commonly be formed in autoimmune disorders. The mechanisms responsible for the latter must involve different mechanisms than those responsible for autoantibodies to the mimicking epitope. (*J. Clin. Invest.* 1995. 95:1316–1327.) Key words: autoimmunity • epitope • virus • scleroderma • lupus erythematosus

Introduction

Epstein-Barr nuclear antigen-1 (EBNA-1)[1] is a nuclear antigen encoded by EBV, and it is universally present in EBV-infected cells. It is critical to viral DNA replication and thus to viral persistence in host cells. It is also a potent antigen, its glycine/alanine (gly/ala) repeat constituting its major antigenic site, as well as a basis for autoantibody production by mimicry (1, 2). We have previously noted in infectious mononucleosis (IM) that IgM autoantibodies are produced to numerous B cell antigens, and that most of them are inhibited by gly/ala synthetic peptides representative of EBNA-1's gly/ala repeat (1). In the preceding paper (2), we reported that one of two recombinant B cell autoantigens recognized by IgM autoantibodies in IM, p542, contains a 28-mer sequence, GGGASGGGGGGGSG-GGGSGGGGGGGSS, which is a mimicking epitope for the gly/ala repeat of EBNA-1. The 28-mer also is the major autoantigenic site in the p542 molecule. Similar Gly-rich epitopes exist in other mammalian and viral proteins, and these may constitute other targets or inducers of anti-p542-like autoimmunity.

Once infected by EBV, one carries the virus for the rest of one's lifetime (3). Since all cells infected by the virus produce the EBNA-1 antigen, EBNA-1 must pose a constant threat of autoimmunization. Here we follow the course of the antibody response to the gly/ala peptide of EBNA-1, together with the autoantibody levels to p542, in students during acute IM and through the following year. We also examine the frequency with which the autoantibody to p542 can be found in normal populations and in autoimmune diseases. IgM anti-p542 continues to be made long after acute IM. IgG anti-p542, however, develops infrequently during this time. IgG anti-p542 is also seen infrequently in normal controls, but it is found in high titer in subpopulations of several autoimmune diseases.

The appearance in multiple autoimmune diseases of IgG anti-p542, an autoantibody that can be attributed to virus infection and which is not explicable simply on the basis of polyclonal B cell stimulation, constitutes a novel finding.

In further studies, we have recognized two autoantigenic epitopes on p542 in addition to its mimicking 28-mer. Examination of one of these epitopes suggests that it has no mimicry with any EBV-encoded antigen, and thus that p542 can be an active autoimmunogen on its own. We suggest that the autoimmunogenicity of this second epitope depends upon preexisting autoantibody to the original mimicking epitope.

Methods

Sera. Students with infectious mononucleosis were volunteers at San Diego State University, San Diego, CA, who agreed to repeated bleedings during their acute illness and in a followup period. Diagnosis of IM was based upon the presence of typical clinical presentation, blood smears, and positive heterophil tests. Viral capsid antigen (VCA)-positive and VCA-negative controls were from 16–17-yr-old volunteer students in La Jolla High School, La Jolla CA. The normal control popula-

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1. *Abbreviations used in this paper:* CMV, cytomegalovirus; EBNA-1, Epstein-Barr nuclear antigen-1; gly/ala, glycine/alanine; IM, infectious mononucleosis; PSS, progressive systemic sclerosis; SjS, Sjögren's syndrome; UC, ulcerative colitis; VCA, viral capsid antigen.

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tions were composed of 29 healthy 20–50-yr-old volunteer hospital workers at Scripps Clinic and Research Foundation, La Jolla, CA, 6 similar volunteers from Cedars-Sinai Medical Center, Los Angeles, CA, an additional 37 from the UCSD Medical Center in San Diego, CA, and 30 healthy ≥ 70-yr-old volunteer Associates of the Sam and Rose Stein Institute for Research on Aging, UCSD. Sera of patients with SLE were provided by Drs. David Horwitz and Francisco Quismorio, University of Southern California Medical Center, Los Angeles, CA, Dr. John Harley, University of Oklahoma Medical School, Norman, OK, and Dr. Harry Bluestein, University of California, San Diego. Sera from patients with progressive systemic sclerosis were from Dr. Thomas Medsger, University of Pittsburgh Medical School, Pittsburgh, PA; Sjögren's syndrome (SjS) from Dr. Robert Fox, Scripps Clinic and Research Foundation; ulcerative colitis and Crohn's disease from Dr. Stephan Targan, Cedars-Sinai Medical Center; ankylosing spondylitis from Dr. David Yu, University of California, Los Angeles; Alzheimer's disease from Dr. Robert Katzsman, University of California, San Diego; and chronic hepatitis B infection from Dr. Frank Chisari, Scripps Clinic and Research Foundation. Sera from patients with rheumatoid arthritis were collected by ourselves from individuals with advanced, long-standing, active disease at Scripps Clinic and Research Foundation. Sera from patients with nondemyelinating neurological diseases were provided by Dr. Patrick Bray (4).

Enzyme linked immunoassays. ELISAs were carried out as previously described (2). In brief, 96-well microtiter trays (Costar Corp., Cambridge, MA) were coated with antigen or peptide at a previously determined optimal concentration (10 μg/ml), blocked with 1% bovine serum albumin, and probed with antibody applied in powdered milk for 1 h at room temperature. The secondary antibody was rabbit anti–human IgM or IgG conjugated with horseradish peroxidase. A 1:100 serum dilution was routinely used, having been shown to be regularly on the downslope of the titration curve and thus to provide a good measure of relative antibody concentration in various sera. For studies of antibodies to deletion mutants of p542, the wells were coated with mutant antigen at 5 μg/ml. Preliminary studies using the mutants at concentrations varying from 3 to 30 μg/ml revealed increasing OD values throughout this range, but only the antibody binding to wells coated at the lower antigen concentrations could be inhibited by soluble antigen at 30 μg/ml or less, and therefore 5 μg/ml was chosen for coating the wells to assay specifically for this inhibitable, higher affinity antibody population.

Western blots. Extracts of bacterial or mammalian cells in Laemmli buffer were prepared as previously described (2). They were electrophoresed in polyacrylamide gels, transferred to nitrocellulose, blocked with powdered milk in borate buffered saline, probed with purified antibody in powdered milk and 10% glycerol, and developed with enzyme-labeled goat anti–human IgG or IgM.

Recombinant autoantigens. The p542 and p554 autoantigens were prepared from the gene fragments, as described in a previous study (2). The β-galactosidase fusion protein, p542-B, was isolated as a 70-kD product after electrophoresis in large acrylamide gels of lysates of bacteria carrying the gene in a pSEM expression vector (2). The hexahistidine fusion proteins, p542-H and p554-H, were isolated on nickel columns from lysates of bacteria carrying the genes in pRSET expression vectors (Invitrogen, Inc., La Jolla, CA). The p542 gene fragment has been sequenced and is being reported separately (Rhodes, G. H., J. R. Valbracht, and J. H. Vaughan, manuscript in preparation).

Preparation of deletion mutants of p542. Deletion mutants of p542 were prepared using the p542 inserts in the pRSET expression vector, so that all mutants had NH_2-terminal hexahistidine fusion partners (Fig. 1). For the D1 mutant, the DNA was cut at an EcoNI restriction site in the insert and a HindIII site downstream in the plasmid, with subsequent blunt ending and religation. For the D2 mutant, the DNA was cut at the ClaI and EcoNI sites within the insert, with blunt ending and in-frame religation. For D3, the gene was cut at ClaI and the downstream HindIII site, with blunt ending and religation. JM109 bacteria were transformed with the respective mutant vectors and grown up with selection media. Expression of the mutant proteins was confirmed in polyacrylamide electrophoresis of the lysed bacteria. For initial evaluation of the reactivities of the mutant products with immunoaffinity-purified antibodies, crude lysates of the respective bacterial transformants were examined in Western blots. For later ELISA studies of serum antibodies, the mutant products were isolated from the lysates by two cycles of absorption and elution from nickel columns, the first elution being carried out and pH 4.0 and the second in 300 mM imidazole at pH 6.3, with final purification by FPLC.

Recombinant EBNA-1. Recombinant EBNA-1 was expressed by transfecting monkey kidney (Cos7) cells in 100-mm petri dishes, using an expression vector containing the SV40 early promoter and the EBNA-1 gene (2). The plasmid was kindly provided to us by Dr. Elliott Kieff (Harvard Medical School, Cambridge, MA).

Immunoaffinity purification of autoantibodies. Recombinant autoantigen was isolated on nitrocellulose strips after transfer from polyacrylamide gel electrophoresis. The excised strips were used for absorption of autoantibodies from serum, as previously described (2). The autoantibody was eluted at pH 11.5 and immediately neutralized in dihydrogen phosphate, 3% powdered milk, and 10% glycerol.

Antigens and synthetic peptides. The antigens were the same as those used in the previous paper (2). The synthetic peptides used here were EBNA-1 gly/ala (P62): AGAGGGAGGAGAGGGAGGAGC; EBNA-$1_{621-635}$: GDDGDDGDEGGDGDE; CMV EA (C2): SSSSAGGGGGGAGGGGGGGGSGG; and p542 21-mer: GGGGGGSGGGGSGGGGGGGSS.

p542 DELETION MUTANTS AND REACTIVITIES

	ClaI EcoNI	IgM SD55	IgM SD41	IgG E90	IgG E77
p542	`---------|---xxxxx---|-------`	+++	+++	+++	++
D1	`-------------xxxxx---`	+++	++	+++	+
D2	`---------          --------`	-	++	++	++
D3	`---------`	-	±	-	-

Figure 1. Diagrammatic display of the mutants prepared from the p542 gene, and the reactivities shown by four immunoaffinity-purified anti-p542 with recombinant products from the mutants. The mutants D1, D2, and D3 were prepared by ClaI/HindIII, ClaI/EcoNI, and EcoNI/HindIII cuts of p542, with blunt ending and religation (HindIII is downstream in the plasmid). xxxxx, Gly-rich sequence. The relative intensities of staining in Western blots shown at the right are also seen in Fig. 6.

Results

Lack of IgG anti-p542 during and after acute infectious mononucleosis. To evaluate the times of appearance of IgM and IgG anti-p542, and of antibody to a gly/ala peptide, P62, representing the mimicked region of EBNA-1, 14 volunteer college students with IM were bled during their acute illnesses and serially thereafter for up to 14 mo. All were heterophile antibody positive. The students remained ambulatory, returning at intervals to make blood donations during followup. Fig. 2, *top*, illustrates the levels of their IgM anti-p542 autoantibodies. These were high in the acute disease and fell somewhat by the third month, but they generally persisted elevated at about this level for the remaining period of observation. This persistence lasted well into the period when most patients were already showing a considerable rise in IgG antibody to the gly/ala (EBNA-1) peptide, P62 (Fig. 2, *bottom*).

Despite the rise in IgG anti-P62 peptide, IgG anti-p542 was infrequently seen (Fig. 2, *middle*). Two students had low levels of IgG anti-p542 during the acute episode, but both lost this by

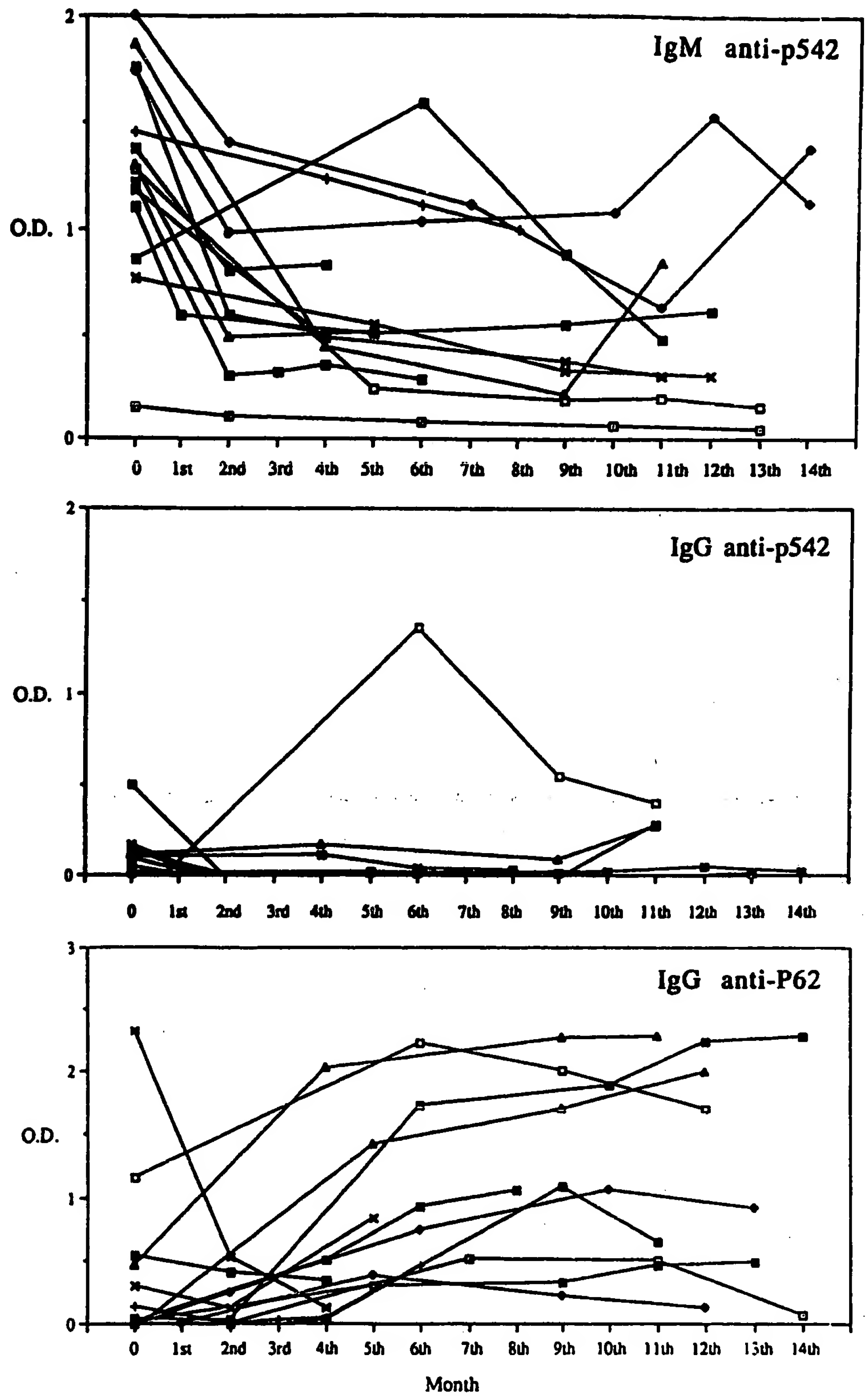

Figure 2. Serial observations of antibody levels by ELISA during and in convalescence from acute infectious mononucleosis. All patients were heterophile positive at initial diagnosis. The individual whose values peaked at 6 mo was the same in all three panels.

the first follow-up bleeding. Both of these students had very much higher IgM than IgG anti-p542 on these occasions. One student developed a striking elevation of IgG anti-p542 at the 6-mo follow-up, with a decline thereafter. This student also had a parallel rise and decline in his IgM anti-p542. Two other students showed small rises in IgG anti-p542 at about a year. On review of the student health clinic records, there were no remarkable symptomatic features, or routine clinical laboratory findings, to match these anti-p542 changes.

IgG autoantibody to a different and nonmimicking autoanti-

Table I. Autoantibody (Anti-p542) and Antibody (anti-p62 and Anti-C2) Titers in Autoimmune Diseases and Controls

Serum sources	No.	IgG Anti-p542H-G					IgG Anti-p62			IgG Anti-C2		
		Mean	SD	SEM	>2 SD	>3 SD	Mean	SD	SEM	Mean	SD	SEM
La Jolla control	29	0.036	0.069	0.013	1 (3.4%)	1 (3.4%)	1.143	0.552	0.102	0.087	0.127	0.023
San Diego control	37	0.014	0.044	0.007	1 (2.7%)	1 (2.7%)	1.264	0.640	0.105	0.021	0.023	0.004
L.A. control	6	0.055	0.126	0.051	1 (16.7%)	0 (0.0%)	1.509	0.250	0.102	0.028	0.061	0.025
≥70 yr control	32	0.049	0.170	0.030	2 (6.3%)	2 (6.3%)	1.498	0.332	0.059	0.051	0.162	0.029
Cord blood	9	0.110	0.253	0.084	1 (11.1%)	1 (11.1%)	0.741	0.573	0.191	0.000	0.000	0.000
VCA negative	15	0.000	0.000	0.000	0 (0.0%)	0 (0.0%)	0.050	0.112	0.029	0.000	0.000	0.000
VCA positive	24	0.068	0.070	0.014	2 (8.3%)	0 (0.0%)	1.274	0.846	0.173	0.050	0.155	0.032
Strept throat	9	0.011	0.020	0.007	0 (0.0%)	0 (0.0%)	1.148	0.586	0.195	0.000	0.000	0.000
IM	20	0.123	0.215	0.049	4 (21.1%)	4 (21.1%)	0.250	0.444	0.099	0.021	0.040	0.009
PSS (Pittsburgh)	40	0.440	0.721	0.114	15 (37.5%)	14 (35.5%)	1.784	0.379	0.060	0.533	0.818	0.129
Ulcerative colitis	12	0.419	0.691	0.199	4 (33.3%)	4 (33.3%)	1.010	0.717	0.207	0.204	0.585	0.169
SLE (Horwitz)	20	0.405	0.575	0.129	10 (50.0%)	9 (45.0%)	0.938	0.811	0.181	0.024	0.078	0.018
SLE (Harley)	86	0.214	0.438	0.047	24 (27.9%)	17 (19.8%)	1.015	0.627	0.068	0.078	0.287	0.031
SLE (Bluestein)	66	0.097	0.274	0.034	7 (10.6%)	6 (9.1%)	1.022	0.641	0.079	0.024	0.049	0.006
Sjögren's syndrome	49	0.256	0.528	0.075	13 (26.5%)	12 (24.5%)	1.094	0.686	0.098	0.098	0.251	0.036
Ankylosing spondylitis	12	0.227	0.260	0.075	4 (33.3%)	4 (33.3%)	1.474	0.216	0.062	0.021	0.032	0.009
Rheumatoid arthritis	37	0.225	0.456	0.075	9 (24.3%)	8 (21.6%)	1.271	0.558	0.092	0.039	0.111	0.018
Crohn's disease	12	0.190	0.378	0.109	3 (25.0%)	3 (25.0%)	1.243	0.633	0.183	0.112	0.320	0.092
Alzheimer	12	0.147	0.182	0.053	3 (25.0%)	1 (8.3%)	1.534	0.165	0.048	0.020	0.051	0.015
Hepatitis B	28	0.101	0.069	0.013	5 (17.9%)	1 (3.6%)	1.242	0.603	0.114	0.017	0.051	0.010
Non-MS neurological disease	15	0.085	0.169	0.044	1 (6.7%)	1 (6.7%)	0.841	0.497	0.128	0.005	0.009	0.002

Underlined means are significantly different ($p \leq 0.05$) from the La Jolla controls. The Alzheimer's disease means were not significantly different from the ≥70 yr controls.

gen, p554, (2) was elevated already at the first bleeding during acute IM in most patients (not shown), indicating that there was no general impairment in the ability to make IgG autoantibodies during the infection.

IgG anti-p542 in autoimmune diseases. The sera from VCA$^+$ and VCA$^-$ 16–17-yr-old high school students, 20–50-yr-old healthy volunteer hospital workers, and 70–90-yr-old healthy volunteers had very little IgG anti-p542 reactivity (Table I and Fig. 3). By contrast, patients with progressive systemic sclerosis (PSS) and ulcerative colitis (UC), had strikingly elevated mean titers of IgG anti-p542; and patients with Crohn's disease, rheumatoid arthritis, Sjögren's syndrome, and ankylosing spondylitis had intermediate mean titers. Three separate series of SLE patients had high, intermediate, and low mean titers (discussed below). Patients with Alzheimer's disease, chronic hepatitis B infection, and nondemyelinating neurological diseases had the least antibody. The anti-p542 data are presented in Table I both as mean titers for each group, and as frequencies with which values ≥ 2 and ≥ 3 SD above the normal 20–50-yr-old controls were found.

In each of the autoimmune groups, many of the sera had IgG anti-p542 levels that actually were negative for the autoantibody, or within the normal range, but subgroups within each had very high levels. This is graphically demonstrated for PSS, SLE, and UC as compared with other groups in Fig. 3, and by the frequencies with which values ≥ 3 SD of the normal mean were seen in these and other autoimmune patients in Table I.

In previous studies (5), we noted that cytomegalovirus (CMV) infection can generate IgM autoantibodies with reactivities in Western blots resembling those of the IgM autoantibodies generated during EBV infection; and we have noted (Fig. 10 in reference 2) that CMV as well as other exogenous agents encode proteins which have glycine-rich sequences which are reasonable candidates for inducing anti-p542 autoantibodies. We wished, therefore, to get some estimate of whether either EBV or CMV might be associated with the IgG anti-p542. Therefore, we assayed by ELISA the titers of antibody to the EBNA-1 gly/ala peptide, P62, and the CMV early antigen peptide, C2 (Table I). Except for PSS, antibody to the CMV early antigen peptide was negative in almost all sera, there being no more than a single positive serum in each disease group. In PSS, however, significant anti-C2 titers occurred in 15/40 (38%) of the sera, the mean for the entire group being 0.533 OD. Within the PSS group there was no relation of the anti-C2 titer to the anti-p542 titer.

Anti-P62 was present in essentially all the patients' sera. Here too, there was no good correlation between the anti-P62 titer and the anti-p542 titer, except that for the vast majority of the sera the anti-p542 titer fell within the anti-P62 titer of the same serum (only 22 of 134 doubly positive sera had anti-p542 titer higher than anti-P62 titer, as opposed to a theoretical 50–50 distribution for unrelated antibodies, X^2 16.3, $P < .0001$, assuming equal sensitivities of the assays). This distribution is consistent with most anti-p542 autoantibodies being subcomponents of the anti-P62 antibodies. This possibility is more directly supported by data shown below in Figs. 4 and 7.

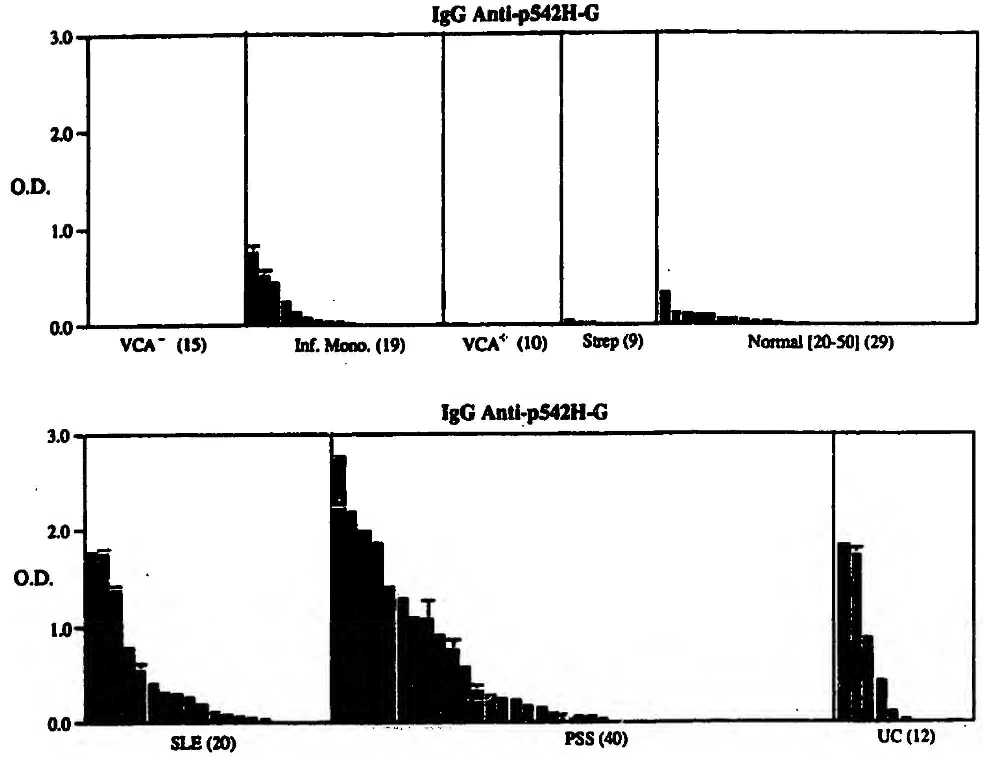

Figure 3. Titers of IgG anti-p542 by ELISA in normal VCA$^-$ and VCA$^+$ teenage controls, normal hospital employee volunteers 20–50 yr old, college students with infectious mononucleosis or strep throats, and patients with systemic lupus erythematosus, progressive systemic sclerosis, or ulcerative colitis. The SLE patients were from Dr. Horwitz (see Table I).

Specificity of IgG anti-p542. Seven SLE, one RA, two IM, and two normal sera were selected for immunoaffinity purification of their IgG anti-p542 autoantibodies. The autoantibodies were adsorbed onto p542-B, a 70-kD β-galactosidase fusion protein of p542 purified by gel separation and electrotransfer to nitrocellulose, as described in Methods. The autoantibodies eluted from this nitrocellulose-bound p542 were tested in ELISA against p542-H, a hexahistidine fusion protein of p542, which had been purified by adsorption and elution from a nickel column. All sera had been preabsorbed with extracts of *E. coli* carrying plasmids with antisense DNA inserts as a precaution against detecting IgG antibodies to *E. coli* products.

The immunoaffinity-purified IgG anti-p542 was highly specific (Table II). There was no cross-reactivity with keratin, which had been a major cross-reacting protein for IgM anti-p542 in our studies in the preceding paper (2), nor with any of the unrelated proteins and peptides tested, with the single exception of an anti-Hsp60 reaction in the normal serum AS47. Two of the seven SLE anti-p542 reacted with the P62 and P60 gly/ala peptides. Thus in ELISA the IgG autoantibodies were highly specific for p542, but in only two cases could they be clearly associated by this method with a gly/ala reactivity.

Anti-EBNA-1 reactivity of anti-p542. The weakness of the reactivity of anti-p542 with the gly/ala peptides in ELISA put in question whether anti-p542 does in fact cross-react with EBNA-1. We, therefore, examined more directly this presumed relationship by testing immunoaffinity-purified preparations of anti-p542 against recombinant EBNA-1. The EBNA-1 was pre-

Table II. Reactivities of Immunoaffinity-purified IgG Anti-p542-B from Various Sera with p542-H and Other Antigens

	Serum sources										
	Normal		R.A	IM		SLE					
Antigens	AS47	VCA + AG	IRA20	SD89	A1M1	E77	E90	E92	SLE13	SLE18	SLE19
p542-H	0.829±.009	0.621±.014	1.735±.078	1.133±.017	0.350±.028	2.024±.034	1.109±.020	0.880±.032	1.752±.053	0.637±.020	1.975±.023
p554-H	0.037±.002	0.075±.013	0.084±.007	0.058±.007	0.061±.009	0.009±.001	0.000±.008	0.000±.001	0.073±.010	0.062±.003	0.062±.003
Keratin	0.058±.011	0.036±.004	0.036±.007	0.055±.013	0.067±.009	0.000±.004	0.000±.014	0.000±.001	0.033±.004	0.055±.005	0.053±.017
p60	0.000±.008	0.060±.003	0.072±.009	0.077±.011	0.070±.013	0.006±.001	0.142±.019	0.012±.001	0.000±.011	0.027±.006	0.275±.005
p62	0.016±.011	0.051±.005	0.058±.008	0.048±.007	0.099±.010	0.016±.004	0.375±.018	0.003±.003	0.000±.007	0.041±.001	0.518±.007
p89	0.006±.040	0.137±0.24	0.130±.009	0.150±.022	0.161±.020	0.000±.001	0.000±.009	0.002±.006	0.024±.022	0.016±.006	0.007±.006
Human collagen II	0.000±.004	0.000±.004	0.000±.007	0.000±.002	0.000±.018	0.006±.018	0.021±.010	0.000±.018	0.000±.006	0.000±.005	0.000±.003
Thyroglobulin	0.139±.009	0.034±.008	0.000±.005	0.081±.006	0.021±.016	0.000±.008	0.000±.010	0.000±.005	0.015±.008	0.027±.007	0.044±.003
Human Hsp60-11	0.216±.028	0.163±.020	0.035±.005	0.150±.016	0.035±.011	0.000±.013	0.000±.010	0.000±.011	0.048±.011	0.074±.007	0.121±.020
groEl	0.011±.006	0.003±.003	0.133±.003	0.000±.004	0.199±.008	0.028±.011	0.000±.006	0.000±.011	0.023±.006	0.003±.002	0.005±.004
Pneumococcus	0.000±.003	0.000±.001	0.000±.001	0.000±.001	0.000±.005	0.000±.003	0.000±.011	0.000±.001	0.000±.008	0.000±.001	0.000±.001
Actin	—	—	—	—	—	0.000±.001	0.000±.008	0.000±.006	—	—	—
Insulin	—	—	—	—	—	0.000±.002	0.000±.012	0.000±.004	—	—	—

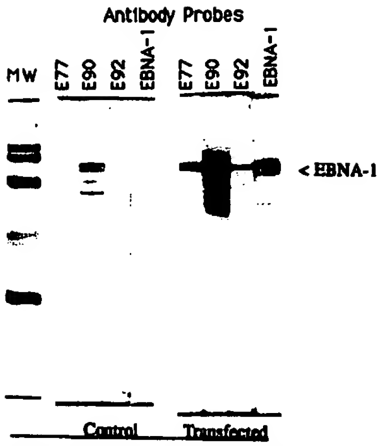

Figure 4. Western blot reactivities of immunoaffinity-purified IgG anti-p542 from SLE sera E77, E90, and E92 probed against an extract of COS-7 cells transfected with EBNA-1 DNA (*right*). Reactivities with an extract of nontransfected COS-7 cells is shown in the left panel. A standard anti-EBNA-1 antibody (G.R.) was used for positive identification in the lane marked EBNA-1. Mol wt (*MW*) markers are at 116, 97, 66, 45, and 29 kD.

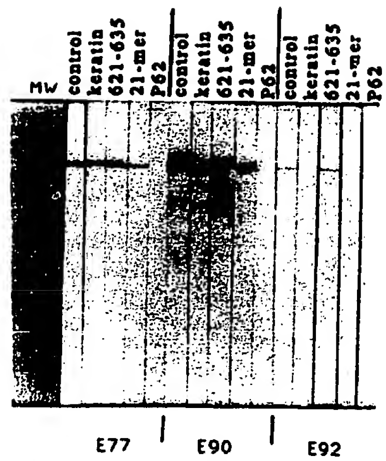

Figure 5. Inhibition of the reactivities of the IgG anti-p542 preparations illustrated in Fig. 4. Keratin, the EBNA-1 peptide 621–635 (see text), a peptide consisting of the C-terminal 21 residues of the p542 28-mer, and the Gly/Ala peptide P62 were preincubated at 10 μg/ml and 37°C for 1 h with the IgG anti-p542 preparations. These, with uninhibited controls, were then used to probe Western blots of the EBNA-1-transfected COS-7 cells. The first set of five lanes was probed with the inhibited and control anti-p542 preparations from E77, the second with similar preparations from E90, and the third from E92. Mol wt (*MW*) markers are the same as in Fig. 4.

pared from a lysate of monkey kidney cells (Cos7) transfected with the EBNA-1 gene (Methods). Extracts of the transfected cells were electrophoresed in polyacrylamide, transferred to nitrocellulose, and probed by the immunoaffinity-purified anti-p542. For controls, nontransfected monkey kidney cells were similarly grown, extracted, and submitted to Western blotting.

Presented in the left panel of Fig. 4 are Western blots with the control cells probed with the anti-p542; in the panel on the right are Western blots with the transfected cells. Lanes *EBNA-1* show the negative and positive reactions of the control and transfected cell preparations probed with a prototype IgG anti-EBNA-1 serum (G. Rhodes). Lanes *1–3* were probed with anti-p542 from three SLE sera and developed with an anti-IgG reagent. All three showed very strong reactivity with the recombinant EBNA-1. Additionally, the anti-p542 from E90 reacted with multiple bands in the nontransfected monkey kidney cells, indicating that autoantigenic configurations similar to ones in the human p542 are present also in this nonhuman primate.

Since anti-gly/ala reactivity had not been prominent in the ELISA data in Table I, the strengths of reactivities of the IgG anti-p542 with the recombinant EBNA-1 in Fig. 4 were at first unexpected. We, therefore, wondered whether these reactions might be based upon mimicking sequences between EBNA-1 and p542 that were additional to the gly/ala-28-mer mimic. We repeated the above Western blots using EBNA-1 as antigen, but preincubated the purified autoantibodies in (*a*) buffer control, (*b*) keratin, (*c*) a synthetic 15-mer peptide from EBNA-1 (residues 621–635) containing a pentamer DDGDE that is common to p542 and EBNA-1 and therefore potentially a second mimicking epitope, (*d*) a synthetic 21-mer peptide representing the downstream residues of the glycine-rich 28-mer in p542, or (*e*) the P62 gly/ala synthetic peptide of EBNA-1. The peptides were used in final concentrations of 10 μg/ml. The P62 peptide completely inhibited the reactions of all three of the autoantibodies with EBNA-1 (Fig. 5), and the 21-mer peptide of p542 was next most potent. Keratin was a weak inhibitor, and the DDGDE-containing peptide was completely inactive. Thus no additional epitopes could be demonstrated. IgG anti-p542 appears to react with EBNA-1 entirely through its gly/ala-28-mer cross-reacting specificity.

Multiple autoepitopes on p542. Although anti-p542 reacts with EBNA-1 purely on the basis of its anti-28-mer reactivity, additional autoantigenic epitopes were not ruled out by this. To look for other epitopes on p542, deletion mutants of the p542 gene were prepared. ClaI and EcoNI restriction sites are present on either side of the glycine-rich 21-mer in p542 (Fig. 1), and they are not present elsewhere in the gene or in the pRSET vector. These therefore were used for preparation of deletion mutants. D1 and D3 mutants were obtained by combining EcoNI and ClaI cuts, respectively, with a HindIII cut downstream in the multiple cloning site of the plasmid, then blunt ending and religation. D2 was a ClaI/EcoNI cut with an in-frame religation. All three mutants were very well expressed, giving excellent bands in Western blots when stained with amido black, and obtained in yields of 800, 255, and 690 μg from nickel columns with 50 ml starting vol of bacterial culture. At the right in the figure are depicted the three patterns of reactivity that we found in Western blots, using immunoaffinity-purified IgM or IgG anti-p542 to probe lysates of bacteria containing the deletion mutants.

Among nine IgG anti-p542 preparations from sera of patients with SLE, all showed reactivity with D1 and most with D2, as illustrated for E77 and E90 in Fig. 6, *bottom*. None showed reactivity with D3. All of four immunoaffinity-purified IgM anti-p542 preparations that had previously shown strong cross-reactivity with keratin (Table II in reference 2) reacted in Western blots exclusively with D1, as shown for SD55 in Fig. 6 *top* left. Both the SD30 and SD41 IgM anti-p542 that had reacted poorly or not at all with keratin (Table II in reference 2) reacted with both D1 and D2 (shown for SD41, *top right*). Both also reacted minimally with D3.

The deletion mutants were purified from the bacterial lysates by adsorption and elution from nickel columns and secondary FPLC to clear them of residual contaminating bacterial protein (as assessed with an anti-*E. coli* reagent, see Methods), and then used in ELISA to assay whole sera for autoantibody activity (Fig. 7). Most sera, whatever their source, that had previously exhibited IgG anti-p542 had IgG reactivity predominantly with the D1 mutant containing the 28-mer. IgG reactivity with the D2 mutant was seen in some sera, but almost always at a lesser titer than shown by the anti-D1 reactivity. IgG anti-D3 reactivity was least commonly seen.

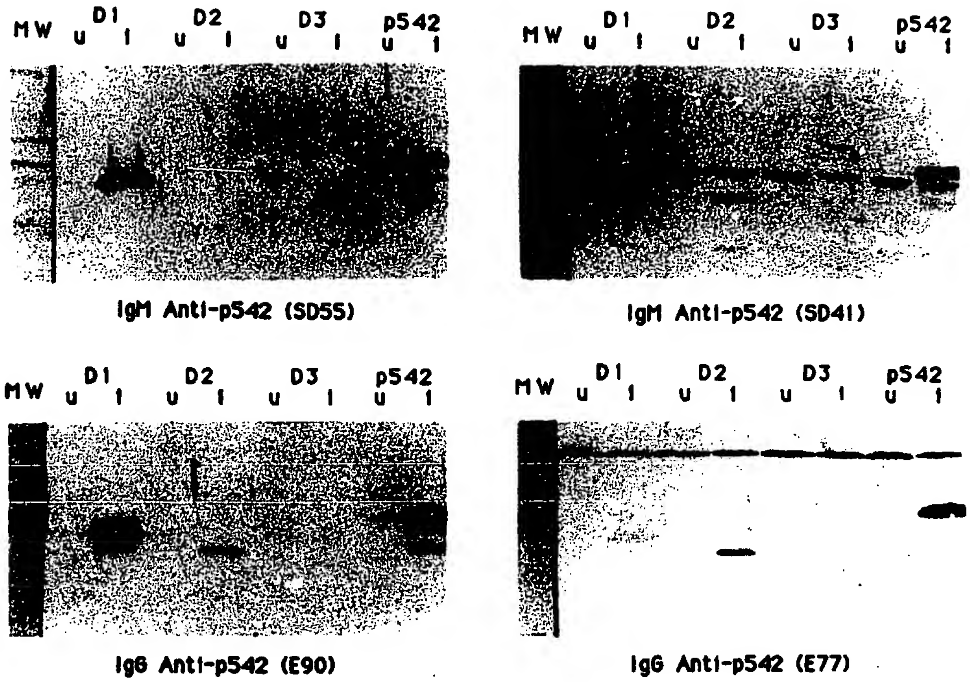

Figure 6. Reactivities of immunoaffinity-purified anti-p542 with deletion mutants (see Fig. 7) in Western blots. Crude lysates of uninduced (*u*) or induced (*i*) bacteria transformed with the pRSET expression vector carrying D1, D2, D3, or p542 inserts were electrophoresed in polyacrylamide. After transfer to nitrocellulose, the strips were probed with the anti-p542 preparations. Bands reactive across all lanes are due to anti-*E. coli* antibodies contaminating the anti-p542 preparations. SD55 reacted with D1 alone. E90 and E77 reacted with both D1 and D2. SD41 shows a very faint reaction with D3, as well as with D1 and D2. Mol wt (*MW*) markers were at 66, 45, 36, 29, and 24 kD.

Anti-D1, -D2, and -D3 during and after acute infectious mononucleosis. We reexamined the reactivities of the sera of the 14 patients shown in Fig. 1 during and after acute infectious mononucleosis for their reactivities with the deletion mutants. High levels of IgM autoantibody reactivity were seen to all three mutants (Fig. 8). The values were high initially and continued to be elevated, but at a somewhat lower level, throughout the follow-up. The strengths of the reactivities followed the general order D1 > D2 > D3. Except for SD30 and SD41, these IgM anti-D2 and anti-D3 reactivities had not been evident in Western blots (see Fig. 8). Since the ELISAs were carried out with whole sera and the Western blots with immunoaffinity-purified autoantibody, we interpret the difference to be due to the presence in the sera of low affinity as well as higher affinity autoantibodies, but with retention of only the higher affinity autoantibodies during the process of immunoaffinity purification.

In the teenage controls (see Table I and Fig. 2), there was also IgM reactivity for all three mutants, and this was true for some VCA$^-$ as well as the VCA$^+$ sera (data not shown).

IgG autoantibody to the mutants was absent or in very low titer in the 14 IM patients. High titered anti-D1 occurred only in a single individual, and only transiently. This was the same individual who had shown the transiently elevated anti-p542 previously (Fig. 2). Lesser rises in IgG anti-D1 occurred at 12 mo in two other individuals, again the same two as for anti-p542 in Fig. 2. There was little or no IgG reactivity with D2 or D3.

Lack of mimicry of the D2 epitope with EBV antigens. IgG anti-D2, which was present in significant quantity in the SLE sera E77 and E90 (see Fig. 6), did not react with recombinant EBNA-1. This was clear from the inhibition studies in Fig. 4, in which the reactivities with EBNA-1 of the anti-p542 in both E77 and E90 were completely inhibited by P62. Thus anti-D2 does not react with EBNA-1.

To test whether anti-D2 cross-reacts with some other EBV-encoded antigen, we reacted immunoaffinity-purified anti-p542 from four sera known to have significant quantities of anti-D2 (one IgM and three IgG autoantibodies) in Western blots with lysates of P3HR1 and B95-8 B lymphocytes before and after their activation by PMA and butyrate to induce virus production. Lysates of the non–EBV-infected B lymphocyte BJAB, also before and after PMA and butyrate, were used as negative controls. The anti-p542 preparations were used as probes with and without inhibition by P62 or D2. In no case did we find the emergence of any new band reactive with anti-D2 in the activated lysates (data not shown).

Discussion

Consistent with our earlier experience in a general analysis of cross-reacting lymphocyte autoantigens by Western blots (1), IgG autoantibody to the recombinant protein p542 was usually very low or undetectable during convalescence from IM, even though IgG anti-gly/ala (anti-EBNA-1) antibodies non–cross-reactive with p542 developed abundantly. The systems that normally disallow switch to IgG of anti-gly/ala autoantibodies in convalescence from IM may include T cell suppressors, specific T cell cytotoxicity, downregulation of specific T cell receptor molecules, and suppression by antiidiotypic antibody or T cells (6–11), and also simple lack of development of anti-p542 specific T cells. Whatever the mechanism, B cells themselves must be central to the process, since the process involves recognition of very precise conformational specificities, i.e., those that differentiate Gly/Ala configurations that are and are not similar to ones present in the p542 28-mer. Antibody alone can do this.

As has been demonstrated for other autoantibodies (12–15), the anti-p542 response probably begins with polyreactive early B cells with low affinity sIgM. For anti-p542, the polyreactivity must include cross-reactivity between configurations in the gly/ala repeat of EBNA-1 and configurations in the Gly-rich 28-mer of p542. Expansion of B cells with this cross-

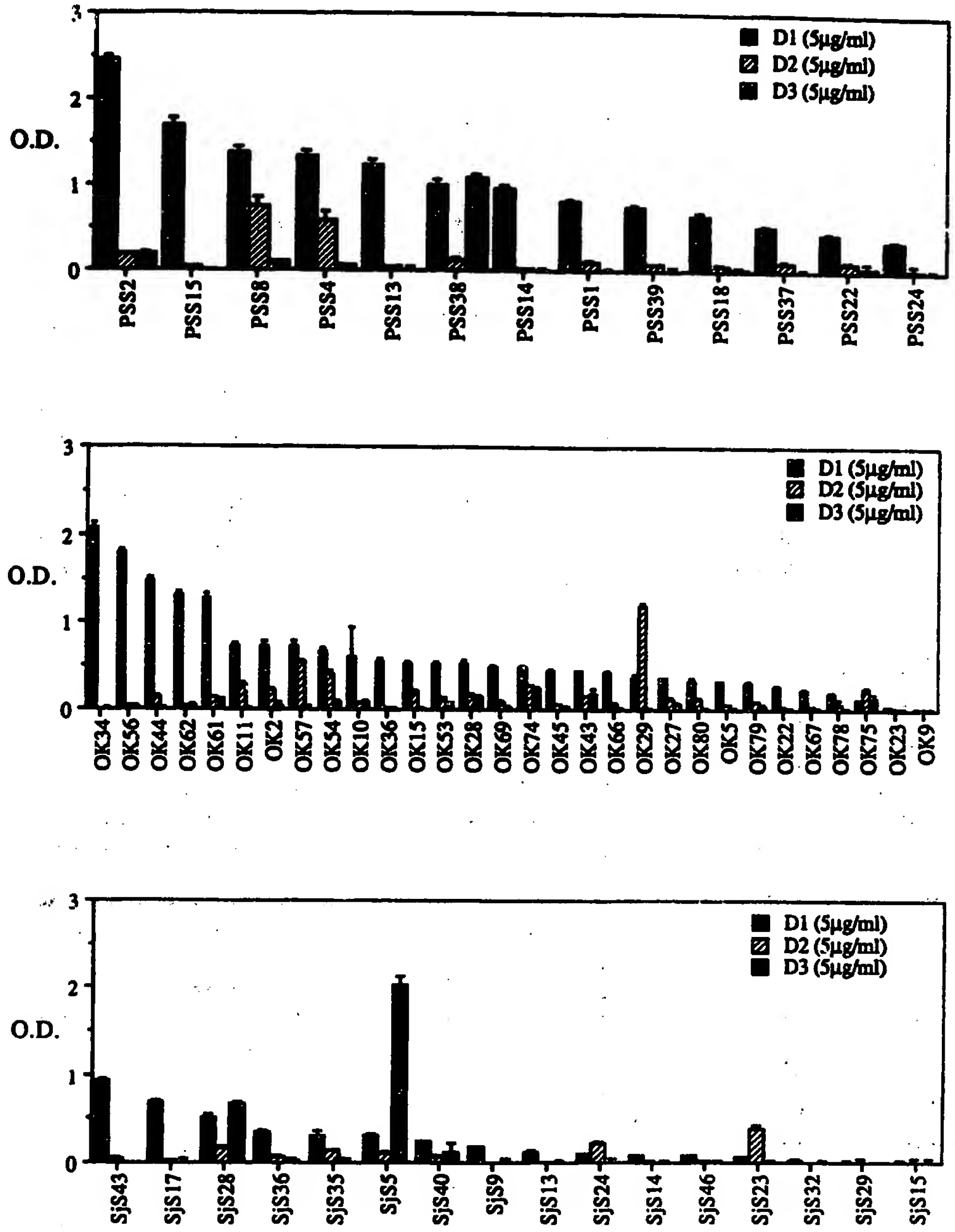

Figure 7. Reactivities in ELISA of anti-p542 positive sera from patients with *PSS*, systemic lupus erythematosus (*OK*), and Sjögren's syndrome (*SjS*) with the D1, D2, and D3 mutants of p542. To eliminate any possible anti-*E. coli* antibodies from the assay, all sera were preabsorbed with lysates of bacteria transformed with the pRSET vector carrying antisense inserts. The mutants were isolated by two cycles of elution from nickel columns (Methods), followed by FPLC, and used at 5 µg/ml to coat the ELISA plates.

reactivity—to give high IgM anti-p542 levels during acute IM—may occur initially by a T cell–independent, but EBNA-1–dependent immunization. (The mechanism for a T cell–independent B cell stimulation could be direct cross-linking of anti-gly/ala sIgM by the EBNA-1 gly/ala repeat. This repeat is poorly or not at all digested by tissue proteases, and it is long enough to display the same epitope several times. The entire P62 sequence, for instance, is present three times in the repeat, and shorter gly/ala sequences appear many more times than that.) The switch from IgM to IgG, however, would require T cell help, and this help must be given precisely to those B cells with p542/EBNA-1 cross-reactivity. Focus on this cell can be brought about only by the B cell's sIg binding the p542 autoantigen itself or, alternatively, EBNA-1 through those gly/ala epitopes of EBNA-1 that are cross-reactive with p542. Subsequent internalization of the p542 or EBNA-1, and presentation of their respective peptides, would allow for T cell response and stimulation of the B cells.

However, anti-EBNA-1–specific T cells have not been detectable in acute IM (16, 17); they emerge only later in convalescence. Anti-p542 specific T cells are probably also very infrequent, although this has not yet been directly investigated. So a strong system for generating an IgM→IgG shift for p542, either through EBNA-1 or through p542, probably does not exist during acute IM. Such a T cell deficiency may alone explain the infrequency of isotype shift, but the effect also could be to make the switch exquisitely sensitive to suppressor factors. Further studies will be needed to differentiate between these possibilities.

Multiple autoantigenic epitopes on p542. The p542 autoantigen has multiple autoepitopes, as is true also for other autoantigens (18–23). The principal epitope is the glycine-rich 28-mer,

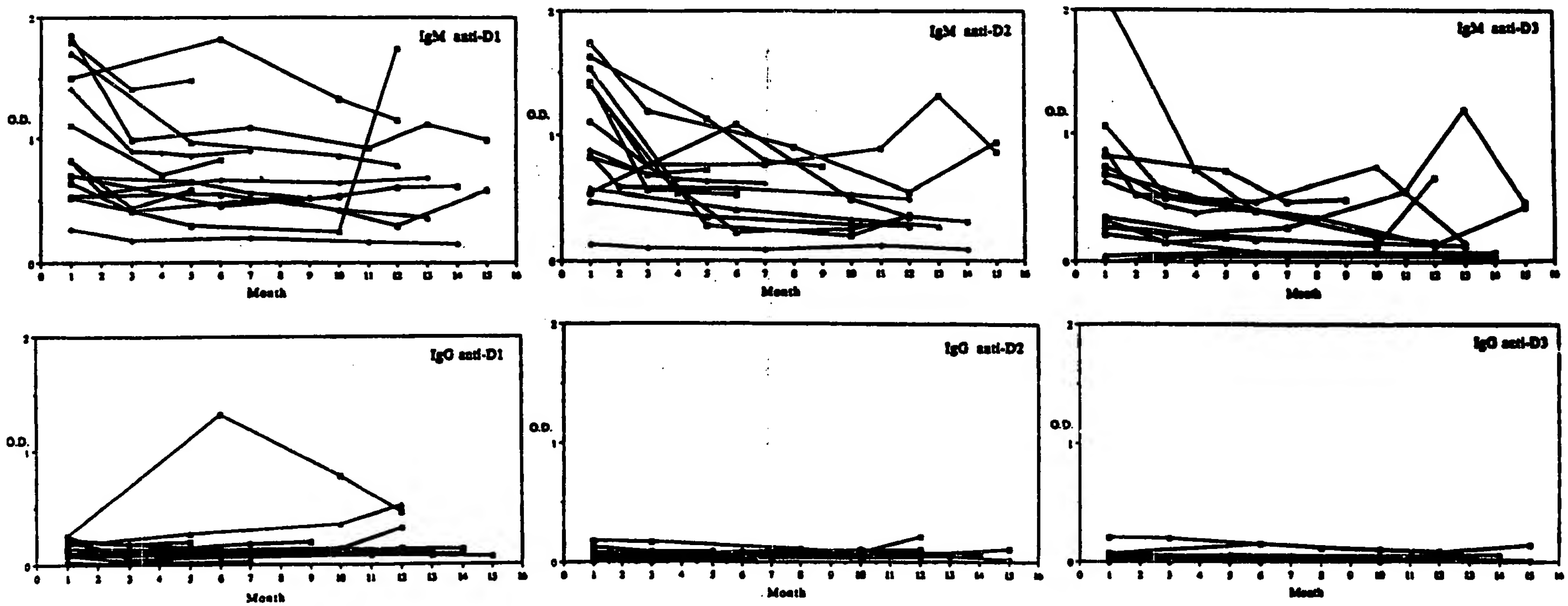

Figure 8. Serial observations on antibody titers to the D1, D2, and D3 mutants of p542 during and after acute infectious mononucleosis. The individuals are the same as in Fig. 2. The assays were by ELISA. The individual showing the high IgG anti-D1 at 6 mos is the same individual who showed a comparable peaking of IgG anti-p542 in Fig. 2.

which is in the D1 deletion mutant. When anti-p542 shifts to IgG, which generally occurs long after acute infection and often in conjunction with some autoimmune disease, the shift always includes autoantibody to the cross-reactive D1. It includes antibody to the non–cross-reactive epitope D2 in about half the sera and to D3 in about 10% of sera. This order in frequency follows the general order of the respective IgM titers of the autoantibodies in acute and convalescent IM sera (Fig. 8), and we interpret this to reflect the relative precursor frequencies for B cells carrying these reactivities.

The autoantigenicity of the D1 epitope is based on its mimicry with EBNA-1. The D2 epitope appears to be non–cross-reactive, neither with EBNA-1 nor with other EBV-generated proteins. Thus the mode of IgG anti-D2 induction must differ from that of anti-D1. The evidence we present here for its non–cross-reactivity is that anti-p542 sera containing IgG anti-D2 did not react detectably with recombinant EBNA-1 through the anti-D2 component (Figs. 4 and 5), nor with extracts of P3HR1 cells that had been induced to productive viral infection by PMA and sodium butyrate. Thus the development of IgG anti-D2 may be an example for autoantibodies of the epitope spreading described by Lehmann et al. (24) for autoreactive T cells in animals immunized with myelin basic protein.

We view that epitope spreading to D2 may operate in the following manner. IgG anti-D2 induction must depend initially upon precursor B cells with low affinity anti-D2 sIgM reactivity. Effective binding of p542 by these anti-D2–specific B cells will not likely be possible, however, except by complexing of the p542 molecule with antibody to the 28-mer, thus generating multivalency for D2. Consequent enhancement of binding, internalization, and presentation of p542 peptides to T cells, affinity maturation, and isotype switch would result. In this scheme, the development of non–cross-reactive anti-D2 is dependent on preexisting cross-reactive anti-D1. This scheme of anti-p542 production is susceptible to testing in an animal model, and it may prove generally descriptive of other autoantibody production.

The scheme assumes that p542 gets into the extracellular space, where p542 could bind anti-D2 reactive B cells. We have so far not detected p542 by flow cytometry as a surface antigen (not shown), and we have not tested for it in extracellular body fluids. There is precedent, however, among other intracellular autoantigens for their sometimes occurring extracellularly, i.e., both myelin basic protein and thyroglobulin have been directly demonstrated in the circulations of normal persons (25, 26). Other observations also are pertinent: (*a*) autoantibodies to intracellular antigens in autoimmune diseases are usually IgG in isotype, which most likely means that autoreactive B cells have picked up the autoantigen extracellularly and then themselves acted as autoantigen-presenting cells to T cells; (*b*) autoantibodies to intracellular as well as extracellular autoantigens exhibit mutational changes characteristic of antigen-driven immune responses (13, 27, 28), which also probably means presentation of extracellular autoantigen; and (*c*) the multiplicity of autoepitopes on most autoantigens is difficult to explain simply by mimicry and probably means that there are potentially autoimmunogenic epitopes on most of them.

The studies of Lin et al. (29), and Mamula et al. (30), strikingly illustrate the importance of extracellular autoantigen in the development of T cell autoimmunity in the mouse. These workers showed that, while human cytochrome *c* injected into mice can induce antibody cross-reactive with mouse cytochrome *c*, there was no accompanying T cell autoimmune response unless mouse cytochrome *c* was also included in the immunizing injections.

IgG anti-p542 in autoimmune diseases. Although IgG anti-p542 was seen infrequently, and only in low titer, in normal control populations, high titers were seen in subsets of several autoimmune diseases. The highest mean titers were in PSS and in UC. Varying mean titers occurred among three SLE groups; and the lowest values were in chronic hepatitis B infection, Alzheimer's disease and nondemyelinating neurological disease controls. We do not yet know definitively why the elevated anti-p542 autoantibody responses occur the in autoimmune diseases, but the skewing of the distribution of titers of anti-p542 towards those of anti-P62 suggests that these two are related to each other.

A simple interpretation that the anti-p542 in these diseases is based on polyclonal B cell activation is not adequate, since all the patient groups had comparable mean titers of antibody to both GroEL and pneumovax antigens, except RA and SjS which were lower (Vaughan, J. H., M.-D. Nguyen, and G. H. Rhodes, manuscript in preparation). Furthermore, the mean titers of these two antibacterial antibodies were not significantly different between sera with abnormally elevated anti-p542 titers (> 2 SD above the normal mean), as compared to those below this level, within each patient group.

One possibility for the elevated anti-p542 levels is that EBV induces the disease in a proportion of patients in each autoimmune syndrome, and that the IgG anti-p542 is a marker of EBV induction in those patients. Alternatively, the IgG anti-p542 is not a marker of a specific inducing agent, but of the fact that each of the diseases has an impaired ability to suppress the expansion of EBV-infected B cells, as has been shown for several of them (31–33), with consequent autoimmunization by resident EBNA-1. A third possibility is that other microbial antigens cross-reactive with p542 may play inductive roles independent of EBV. Finally, the appearance of IgG anti-p542 may be determined by whether the individual's HLA class II molecules are capable of presenting the appropriate p542 peptides for T cell help.

PSS, which had the highest anti-p542 titers, is a disease that is characterized by antitopoisomerase 1, antinucleolar, anticentromere, and antifibrillarin autoantibodies, among others (34). Of these, fibrillarin has in it a glycine-rich 22-mer (35) with 77% identity to p542. We have not yet determined whether this homology is the basis for the high anti-p542 or high anti-C2. Neither EBV nor CMV has generally been considered to be an inducing agent in PSS, nor has any other infectious agent been so considered. However, in view of the close relationship that anti-p542 has to antibody to the EBNA-1 gly/ala peptide P62, and the independently elevated antibody to the CMV peptide C2, this tradition perhaps should be reexamined.

The number of sera of patients with ulcerative colitis we have examined is too small for conclusions to be drawn about our findings. Nevertheless, it is of note that Farmer et al. have reported high anti-CMV titers in UC blood (36), and Wakefield et al. described the detection by nested polymerase chain reactions of EBV and CMV DNA in 16 and 17, respectively, of 21 biopsies of UC colonic tissue (37). Our additional finding in this small series of high anti-p542 titers encourages further investigation in the area.

The three SLE series brings up other important considerations. Anti-p542 was elevated (> 3 SD above the normal mean)

in subsets of all three series of patients more frequently than in the controls, but the numbers of individuals in these subsets varied greatly within each series. The Los Angeles sera were obtained from patients who were hospitalized because of active disease and who had high antinuclear antibody titers. The Oklahoma and San Diego sera were obtained from both inpatient and outpatient sources, and without regard to the activity or inactivity of their disease. The San Diego patients were predominantly Black and Hispanic, the Oklahoma patients predominantly European in origin. At present, we do not know which, if any, of these differences were determinant of the frequencies with which the elevated anti-p542 levels were found.

Nor do we know whether differences in environmental exposure were determinant in the SLE groups, including not only exposure to CMV or other non-EBV viruses, but also exposure to various strains of EBV with differing gly/ala chain length differences (38–40), which we have associated with different cross-reactive potentials (see Fig. 7, reference 2). Antibody titers to EBV antigens and peptides are elevated in SLE (41–47), however, and the elevations are highly selective among EBV antigens (41, 42), as well as exclusive of some other viral species (47). So the elevations of anti-EBV antibodies are not explicable simply on polyclonal B cell stimulation. It remains possible that EBV is a significant, but not exclusive, factor in initiating or compounding this disease.

Finally, we have not followed any of these SLE patients longitudinally to determine the effects of treatment, or variation in disease activity, on their anti-p542 titers.

Among the remaining patient groups, the universally low values of IgG anti-p542 in the chronic hepatitis series document that elevations of this autoantibody are not simply the result of chronic inflammation. The intermediate levels in Sjögren's syndrome, ankylosing spondylitis, and rheumatoid arthritis were, as with the PSS, UC, SLE, and SjS patient groups, each due to subsets of patients with high anti-p542 titers. The mechanisms leading to this development of subgroups, and the implications for pathogenesis in the autoimmunities, will be important areas for future exploration.

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Barr virus infection in young patients suggests a possible etiology for **systemic lupus** erythematosus

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An Increased Prevalence of Epstein-Barr Virus Infection in Young Patients Suggests a Possible Etiology for Systemic Lupus Erythematosus

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Abstract

An unknown environmental agent has been suspected to induce systemic lupus erythematosus (lupus) in man. Prompted by our recent immunochemical findings, we sought evidence for an association between Epstein-Barr virus infection and lupus. Because the vast majority of adults have been infected with Epstein-Barr virus, we chose to study children and young adults. Virtually all (116 of 117, or 99%) of these young patients had seroconverted against Epstein-Barr virus, as compared with only 70% (107 of 153) of their controls (odds ratio 49.9, 95% confidence interval 9.3–1025, $P < 0.00000000001$). The difference in the rate of Epstein-Barr virus seroconversion could not be explained by serum IgG level or by cross-reacting anti-Sm/nRNP autoantibodies. No similar difference was found in the seroconversion rates against four other herpes viruses. An assay for Epstein-Barr viral DNA in peripheral blood lymphocytes established Epstein-Barr virus infection in the peripheral blood of all 32 of the lupus patients tested, while only 23 of the 32 matched controls were infected (odds ratio > 10, 95% confidence interval 2.53-∞, $P < 0.002$). When considered with other evidence supporting a relationship between Epstein-Barr virus and lupus, these data are consistent with, but do not in themselves establish, Epstein-Barr virus infection as an etiologic factor in lupus. (*J. Clin. Invest.* 1997. 100:3019–3026.) Key words: autoimmunity • autoantibodies • viral infection • autoimmune disease • SLE

Introduction

Systemic lupus erythematosus (lupus) is an idiopathic disease characterized by variable inflammatory destruction of skin, joints, blood elements, kidneys, serosa, nervous system, and other tissues (1). In addition, the presence of autoantibodies, as manifest most consistently by a positive antinuclear antibody test, are nearly universal in lupus. A variety of autoantibodies are found in the serum of lupus patients, and constitute evidence that lupus is an autoimmune disease.

We have sought an etiology for lupus based upon the assumption that an understanding of the structural relationships between antigen and antibody would lead towards the immune responses that we have presumed are responsible for initiating lupus autoimmunity. Autoantibodies against the spliceosome, also referred to as anti-Sm and anti-nRNP, are common in lupus, being found in ~ 25–40% of patients. Autoantibodies binding the peptide PPPGMRPP, derived from the amino acid sequence of Sm B/B′, appear to be among the earliest to develop in the humoral autoimmune response against the B/B′ protein of the Sm antigen (2). Immunization with this peptide induces lupus-like autoimmunity in animals (2), as does immunization with the closely related sequence PPPGRRP, found in the Epstein-Barr nuclear antigen-1 (EBNA-1; 3).[1]

Perhaps these structural and immunogenic relationships between EBNA-1 and the fine specificity of the autoantigenic history of the spliceosome in lupus reflect an etiology of lupus. It is possible that an immune response directed against PPPGRRP of EBNA-1 develops antibodies that cross-react with PPPGMRPP in the spliceosome, inducing lupus autoimmunity in some patients after a molecular mimicry mechanism.

If the hypothesis that Epstein-Barr virus is a possible etiology for lupus were true, there must be an association between Epstein-Barr virus infection and lupus. Such an association would not prove this hypothesis; however, the absence of an association would disprove the hypothesis. The lifelong latency of this virus after infection and the constant immune stimulation deriving therefrom make this virus an intriguing candidate for an environmental agent capable of inducing lupus. The high prevalence of Epstein-Barr infection in adults (~ 95% have been infected and are presumed to carry the virus [4]) would mean that other factors must also be important if Epstein-Barr virus was shown to be a required participant.

To have sufficient statistical power to determine whether the predicted association between Epstein-Barr virus and lupus exists, the experiments were performed in children and young adults (all < 20 yr of age) where the frequency of virus infection in normal individuals is substantially lower than that found in the adult population. In the United States, about half of the population is infected with Epstein-Barr virus between the ages of 10 and 20 yr (4).

We are not the first to explore a relationship between Epstein-Barr virus and lupus. The first serious attempt was published over a quarter century ago (5). Despite the dramatically less sensitive and specific methods available at that time, a higher level of antivirus antibody was found in these patients than in the controls. The ratio of patient to control titer was 6.14:1 ($P < 0.001$ from data in reference 5). Some of these same authors found inconsistent results in subsequent studies published in 1973 (6, 7). Since then, the prevailing opinion has

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1. *Abbreviations used in this paper:* EBNA, Epstein-Barr virus nuclear antigen; EBV-VCA, Epstein-Barr virus nuclear antigen viral capsid antigen antibodies; ISR, international standardized ratio.

been that lupus is not directly related to Epstein-Barr virus infection. We reexamine this question using the much more reliable technologies now available, and an experimental design chosen to improve statistical power. The results obtained demonstrate an association between lupus and Epstein-Barr virus infection sufficiently powerful to suggest that Epstein-Barr virus infection is in some way related to the vast majority of lupus cases.

Methods

Patients and controls. All subjects were between 4 and 19 yr of age. Lupus patients satisfy the 1982 criteria for lupus (1). The Oklahoma City lupus patients and controls were similar by age (15.79±2.15 and 15.40±2.51 yr), race (European-American, 53 and 59%; African-American, 27 and 23%; Hispanic, 10 and 9.5%; and other, 10 and 8.5%), and sex (85 and 82% female). Controls in the San Diego group were selected from siblings. For the matched case control study, controls were matched for age (±2 yr), sex, race, and social situation (cases selected controls when possible). In addition, we have two collections of children with other rheumatic diseases, juvenile rheumatoid arthritis, and myositis. These serum samples were generously provided by Dr. Morris Reichlin and the Childhood Myositis Heterogeneity Study Group.

Serologic assays. Antinuclear antibody assays, antispliceosomal antibodies, antispliceosomal antibody absorptions, and IgG levels were performed as previously described (8–10).

Antiviral assays were performed as instructed by Wampole Laboratories (Cranberry, NJ), or for retesting by Gull Laboratories (Salt Lake City, UT). The assay methods and analysis are presented as units of the international standardized ratio (ISR), which is designed to be as accurate as possible for precise detection of seroconversion. Since the ISR is linear with the optical density, the higher levels of antibody will be relatively underestimated. Nevertheless, the ISR is a semiquantitative measure of the relative level of antibody.

Epstein-Barr virus DNA detection. The Epstein-Barr virus DNA assay was modified from previous methods (11, 12) using peripheral blood mononuclear cell DNA and the PCR with the forward 5′-CCAGAGGTAAGTGGACTT-3′ and reverse primers 5′-GACCGGTGCCTTCTTAGG-3′. A positive result produced a 122-nucleotide (nt) DNA fragment that was sequenced and found to be identical to the previously published sequence (position 14,614–14,735, Genbank accession no. V01555). PCR products were Southern-blotted with a [^{32}P]dCTP-labeled probe corresponding to positions 14,639–14,676. This fragment was cloned into pCRII and sequenced using the dideoxy method (13). The sequence obtained matched the expected DNA sequence exactly. Flanking primers (position 14,557–14,574 and 14,759–14,776) were used to confirm results.

For every specimen from which DNA was isolated, six PCRs were evaluated, each containing 2 μg of mononuclear cell DNA. Reactions contained 50 mM KCl, 10 mM Tris-HCl at pH 8.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.2 mM each of dNTP, 0.5 μM primer, and 2.5 U Taq DNA polymerase. Hot-start protocol was performed using Ampliwax PCR Gems (Perkin-Elmer, Branchburg, NJ). The cycles used were as follows: 2 min at 95°C, 1 min at 59°C, 1 min at 72°C (twice); 2 min at 94°C, 1 min at 58°C, 1 min at 72°C (twice); 1 min at 94°C, 1 min at 57°C, 45 s at 72°C (31 times); and 5 min at 72°C.

All samples that were negative for Epstein-Barr virus DNA by the first set of primers were tested for expansion of a 132-nt fragment of 60 kD Ro: (forward) 5′-CATGAAATGTGGCATG TGGG-3′ and (reverse) 5′-AGATCTTTGTGAGACCAGCC-3′. The expanded fragment was present in every sample tested.

All samples negative for Epstein-Barr virus DNA were also tested by PCR for a unique set of polymorphisms from the M13 Map Pair primers from D1S1589, D2S1328, D6S1027, D1S1678, D18S535, and DXS6810 (Research Genetics, Huntsville, Alabama). PCR products were separated, and DNA sequences were obtained on a model 4000L DNA sequencer (LI-COR, Lincoln, NE).

Statistics. The Breslow-Day test was used to test for homogeneity of odds ratios by site of subject colection. The mid *P* exact confidence intervals for the odds ratios were calculated using EXACT (Version 2.0b). The probability of the observed frequencies of seroconversion was calculated by the Fisher exact test. SAS programs (Sas Institute, Cary, NC) were used for stepwise logistic regression. Where normal distributions could not be assumed, nonparametric testing, including median, 25–75% interquartile ranking (IQR), and Wilcoxan Rank test of the means were also calculated by the SAS programs. The binomial test was used to calculate probabilities for the discordant matched cases and controls. To estimate odds ratios when relevant zero cells were encountered, one was added to all cells.

Results

Anti–Epstein-Barr virus viral capsid antigen antibodies (anti–EBV-VCA). To test for an association between lupus and serologic conversion against Epstein-Barr virus, serum (or plasma) from young lupus patients was collected in Oklahoma City. Local controls were assembled to reflect the age, racial composition, and sex of the cases. The preliminary analysis of the cases and controls collected in Oklahoma City suggested that an association between lupus and anti–EBV-VCA antibodies was present. This finding was confirmed as the collection was enlarged (Table I).

Confirmation of this association was also sought by repeating the experiment in a second group of cases and controls. A previously assembled collection of sera from families with a pediatric lupus proband from the San Diego area was evaluated for Epstein-Barr virus seroconversion by the anti–EBV-VCA assay. Sera simultaneously collected from the siblings of the San Diego probands were used as controls.

The young lupus patients from both the Oklahoma City and San Diego cohorts have a much higher rate of EBV-VCA seroconversion than do their respective controls (Table I). Indeed, the Breslow-Day test suggests that the EBV-VCA seroconversion rates of patients and controls collected in Oklahoma and California are statistically homogeneous ($P > 0.05$), and therefore may be combined.

Of the entire collection of 117 young lupus patients, 116 have seroconverted against EBV-VCA (Table I). Meanwhile,

Table I. Seroconversion Against Epstein-Barr Virus Viral Capsid Antigen (EBV-VCA) in Sera from Young Lupus Patients and Controls

	Oklahoma City	San Diego	Combined*
	No. pos (total)	*No. pos (total)*	*No. pos (total)*
Lupus patients	59 (59)	57 (58)	116 (117)
Normal controls	64 (95)	43 (58)	107 (153)
Odds ratio			49.9
95%-CI of O.R.			9.3, 1025
Probability‡			0.0000000000421

Sera from lupus patients (cases) or their controls were tested for IgG anti–EBV-VCA antibodies and standardized for seroconversion. No. pos, number seroconverted. *Since the Breslow-Day test for homogeneity of the odds ratios shows that they are not statistically different in the Oklahoma City and San Diego collections of patients and controls, the two groups have been combined for analysis. ‡Fisher's exact test.

only 70%, or 107 of 153 of the controls, have seroconverted against EBV-VCA. In these patients and controls, Epstein-Barr virus seroconversion is very closely associated with lupus, with an odds ratio of 49.9. The 95% confidence interval for the odds ratio is quite broad (9.3–1035), suggesting that while the association between EBV-VCA seroconversion is convincing, knowing the true magnitude of the association will require a much larger collection of patients and controls. The likelihood that the distribution observed could have occurred by chance is, however, vanishingly small ($P < 0.00000000000421$).

Anti–EBV-VCA IgM was tested in 23 lupus patients' sera and 22 controls to assess if children with SLE have an abberrant IgM-EBV response, or if seronegative controls have been recently exposed to EBV. No anti–EBV-VCA IgM was found in any of these sera. These results suggest that of those who have anti–EBV-VCA IgG evidence of seroconversion, infection with Epstein-Barr virus was in the immunologically distant past.

We have evaluated two groups of control sera from children with other autoimmune rheumatic diseases. 38 children with juvenile rheumatoid arthritis have been tested for IgG binding to EBV-VCA. 29 of the 38 children (72.1%) have evidence of previous exposure to EBV. We have tested 36 childhood myositis patients' sera for anti–EBV-VCA antibodies. 26 of 36 children (72.2%) with clinical and serological evidence of myositis had antibodies to EBV-VCA. Neither of these rheumatic disease subsets showed a significantly greater incidence of EBV exposure than did sera from the two groups of normal control children presented above. An association between either childhood myositis or juvenile rheumatoid arthritis and seroconversion against Epstein-Barr virus would appear to be unlikely, especially at the level found in the two cohorts of young lupus patients presented above.

The powerful association of Epstein-Barr virus seroconversion with lupus has the potential to reorient much thinking concerning the origin of lupus unless the association is explained by some relatively trivial issue. A series of experiments were therefore performed to assess the potential that this close association could be the result of an experimental artifact. We measured the extent of autoantibody cross-reaction with EBV-VCA for the specific example of the spliceosome (Sm/nRNP) by absorption. IgG levels were compared between lupus patient and normal control sera in an effort to assess a possible contribution of hypergamma-globulinemia. In addition, seroconversion against four other herpes viruses was measured. Finally and most importantly, a technically independent assay was developed and applied that directly detected the presence of Epstein-Barr virus DN/. in lupus cases and their matched controls. All of these experiments supported a close association between Epstein-Barr virus infection and lupus, as presented below.

Antispliceosomal antibody absorption. Cross-reactivity between EBV-VCA and the spliceosome was evaluated by testing for anti–EBV-VCA antibodies before and after absorbing the antispliceosomal autoantibodies from five anti–Sm precipitin–positive lupus sera. These sera were specifically selected because their primary autoantibody responses were directed against the spliceosomal proteins. Absorption reduced the antispliceosome activity as measured by binding to the purified bovine nuclear ribonucleoprotein (the U1 particle) by at least 90%, and reduced the fluorescent antinuclear antibody binding by an average of 97% (thereby supporting the contention that the spliceosome is the major autoantigen in these particular anti–Sm precipitin–positive patients). Absorption reduced the IgG anti-EBV in these sera by an average of 8%, but did not alter the EBV seroconversion status of any of these anti–Sm precipitin–positive lupus sera (supporting the absence of a quantitatively relevant level of IgG cross-reactivity between EBV-VCA and the spliceosome).

IgG levels. Nonspecific binding is sometimes found in lupus sera. One explanation for positive results as a result of nonspecific binding is the hypergammaglobulinemia found in some lupus sera. IgG levels were assessed in 28 of the lupus sera and in 28 of the control sera. These control sera were primarily siblings of the lupus probands. The IgG levels were not different between these groups, nor did the IgG level correlate with the level of anti-EBV-VCA (data not presented). The mean level of IgG in lupus patient sera may have been lowered by the inclusion of many patients who had been treated with high-dose prednisone (> 40 mg/d) and cytotoxic agents. The level may have been increased in the controls, because most of those selected (19 of 28) were relatives of lupus patients.

Alternative EBV-VCA preparation. To be certain that the binding to EBV-VCA by lupus sera was not an artifact of the particular EBV-VCA preparation, a second source of this antigen was used. We re-tested 32 lupus sera and 47 control sera. In the lupus patients, the two assays for Epstein-Barr virus seroconversion produced identical results. There were only minor differences in the control sera where three that had earlier been equivocal, and hence seronegative, were anti–Epstein-Barr virus–seropositive in this second assay. This experiment confirmed the unexpectedly high frequency of Epstein-Barr virus seropositivity in lupus patients (odds ratio = 19.24; 95% confidence interval [CI] = 3.06–121.1).

Seroconversion against other herpes viruses. The Oklahoma City and San Diego cases and controls were evaluated for IgG against four other herpes viruses: cytomegalovirus, herpes simplex virus types 1 and 2, and varicella zoster virus. The differences in the seroconversion rates against these viruses between cases and controls (Table II) were much smaller than those ob-

Table II. Seroconversion Frequencies in Pediatric Lupus and Controls for IgG Binding to Cytomegalovirus Antigen (CMV), Herpes Simplex Type 1 Antigen (HSV-1), Herpes Virus Type 2 Antigen (HSV-2), and Varicella Zoster Virus (VZV) Antigens

	CMV	HSV-1	HSV-2	VZV
Oklahoma City				
Lupus patients	24 (59)	39 (59)	27 (59)	56 (59)
Normal controls	28 (95)	43 (96)	28 (96)	87 (96)
San Diego				
Lupus patients	18 (58)	33 (58)	32 (58)	46 (58)
Normal controls	12 (57)	31 (57)	23 (57)	45 (57)
Odds ratio	1.57	1.71	2.03	1.08
CI-95% of odds ratio	0.93, 2.65	1.05, 2.79	1.24, 3.34	0.53, 2.24
Probability	0.11	0.036	0.0059	0.86

Sera from lupus patients (cases) or their controls were tested for IgG antiviral antibodies and standardized for seroconversion. The odds ratios were calculated after combining both groups since the Breslow-Day test shows that the individual odds ratios are homogeneous.

served against Epstein-Barr virus (Table I). The odds ratios for a seroconversion rate difference for cytomegalovirus and varicella zoster did not differ significantly from unity. There were potentially significant differences between patients and controls for herpes simplex virus types 1 and 2.

For herpes simplex type 1, the Breslow-Day test shows that the Oklahoma City and San Diego results are not sufficiently different to require separate analyses. This outcome may be the result of a relatively small sample size of the groups being tested, since the Oklahoma City collection has an odds ratio of 2.4 (nearly twice that of 1.1 found in the San Diego collection). In any case, this seroconversion difference is unlikely to be important since it is of relatively small magnitude and is not consistently found in both collections of patients and controls.

On the other hand, there is a higher rate of seroconversion against herpes simplex virus type 2 in the cases than is found in the controls from both sites. Also, the odds ratios are similar, and the cumulative significance is consistent with this being a difference between young lupus patients and controls in both communities. Relative to the odds ratio for an association of lupus with Epstein-Barr virus (odds ratio = 49.9), however, the odds ratio for an association with herpes virus type 2 (odds ratio = 2.03) is small (Tables I and II).

To determine whether there might be an independent association of lupus with Epstein-Barr virus and herpes simplex virus type 2, stepwise logistic regression was applied to these data using lupus as the dependent variable and the seroconversion status of all five viruses as independent variables. Epstein-Barr virus is incorporated into the model first (odds ratio = 49, score $\chi^2 = 39$, degrees of freedom [df] = 1, $P = 0.0000000005$). The residual χ^2 is so small ($\chi^2 = 6.8$, df = 4, $P = 0.15$) that no further model building is indicated. In these cases and controls, the association between lupus and Epstein-Barr virus is 10^8-fold more significant than it is between lupus and any of the other viruses evaluated. Consequently, beyond the effect of Epstein-Barr virus, none of the other viruses make a meaningful contribution to the overall model, and the high level of Epstein-Barr virus seroconversion cannot be explained by the presence of nonspecific antiviral antibodies.

Levels of antiherpes virus antibodies. The specific association between Epstein-Barr virus seroconversion presented above (Table I) is dependent upon the quantitative levels of antibody only to the extent that the threshold for seroconversion has or has not been exceeded.

The ISR difference of anti-EBV-VCA antibodies between the lupus patients and their controls is impressive: 4.30 vs. 1.92 ISR ($Z = 10.69$, $P = 1.13 \times 10^{-26}$). The differences in the levels of the other antiherpes antibodies is much smaller (Fig. 1). Of these, only antibodies against the herpes simplex virus types 1 and 2 appear to achieve significance ($Z = 3.59$, $P = 0.00034$; and $Z = 5.41$, $P = 6.1 \times 10^{-8}$, respectively), again in concert with the results obtained above by assessing seroconversion rates.

Epstein-Barr virus DNA assay. In aggregate, the data presented above support a specific association between Epstein-Barr virus seroconversion and lupus. To test rigorously the prediction that Epstein-Barr virus infection is associated with lupus, a matched case control study has also been performed using the presence of DNA from Epstein-Barr virus in peripheral blood mononuclear cells as evidence for Epstein-Barr vi-

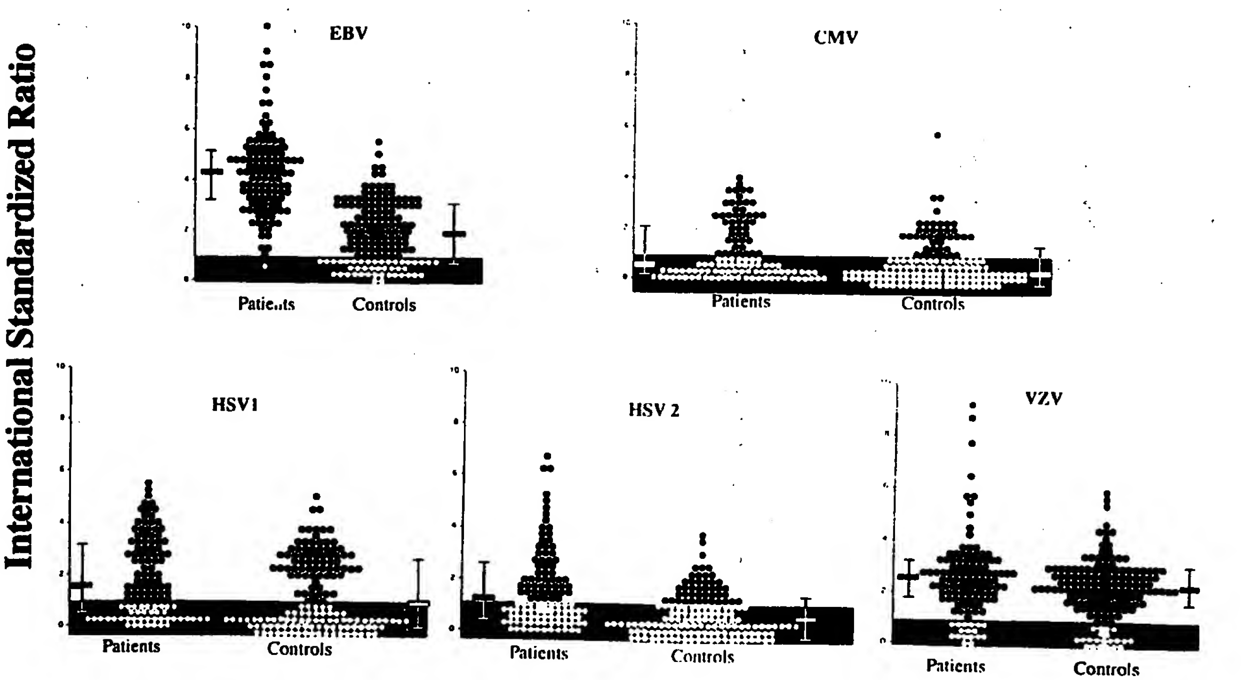

Figure 1. The level of antiviral antibodies. Assays for IgG binding to EBV viral capsid antigen, (EBV), cytomegalovirus (*CMV*), herpes simplex types 1 and 2 (*HSV-1* and *HSV-2*), and varicella zoster virus (*VZV*) are presented for the young lupus patients and their controls, as indicated. The level of antibody is given as ISR. The median and 25–75% IQRs for the lupus patients and controls are for anti-EBV-VCA 4.30 (1.85) and 1.92 (2.20); for anti-CMV 0.617 (1.74) and 0.508 (1.29); for anti-HSV-1 1.56 (2.58) and 0.924 (2.30); for anti-HSV-2 1.16 (1.64) and 0.557 (1.17); and for anti-VZV 2.53 (1.40) and 2.39 (1.28).

rus infection. A very sensitive assay for Epstein-Barr virus DNA was developed based on previous work (11, 12) and by exploiting a nucleotide sequence that is repeated eleven times in the Epstein-Barr virus genome (11).

A total of 32 lupus cases and their matched controls were evaluated for Epstein-Barr virus infection. We asked each case to provide a control matched for age (±2 yr), sex, race, and social circumstances. Seven siblings, 4 cousins, and 12 friends were provided. For those cases that did not provide matched controls, the investigators recruited a matched control (at enrollment, the Epstein-Barr virus seroconversion status of controls was unknown to the investigators). Epstein-Barr virus DNA was assayed in a total of 12 μg of mononuclear cell DNA divided into six reactions from each case and control (Fig. 2). Cases and their matched controls were either concordant (Fig. 2 *A*) for the presence of Epstein-Barr virus DNA, or were discordant (Fig. 2 *B*) where Epstein-Barr virus DNA was found in one or the other, but not in both. Control experiments demonstrate that DNA is present and unique from each individual in whom Epstein-Barr virus DNA was not detected. Examples of these data are presented in Fig. 2, *C–E*.

All 32 lupus cases tested had Epstein-Barr virus DNA. Of the 32 matched controls, 23 had Epstein-Barr virus DNA. In each of the nine discordant pairs, the lupus case had Epstein-Barr virus DNA while the matched control did not (odds ratio > 10, CI-95% 2.53–∞, $P < 0.002$; Table III).

All 32 of the lupus cases were seropositive for Epstein-Barr virus, as were 23 of the matched controls. Epstein-Barr virus DNA was demonstrated in the peripheral blood mononuclear cells in every Epstein-Barr virus–seropositive lupus case and control (n = 55). Two controls were Epstein-Barr virus–seronegative, and yet had Epstein-Barr virus DNA. One of these

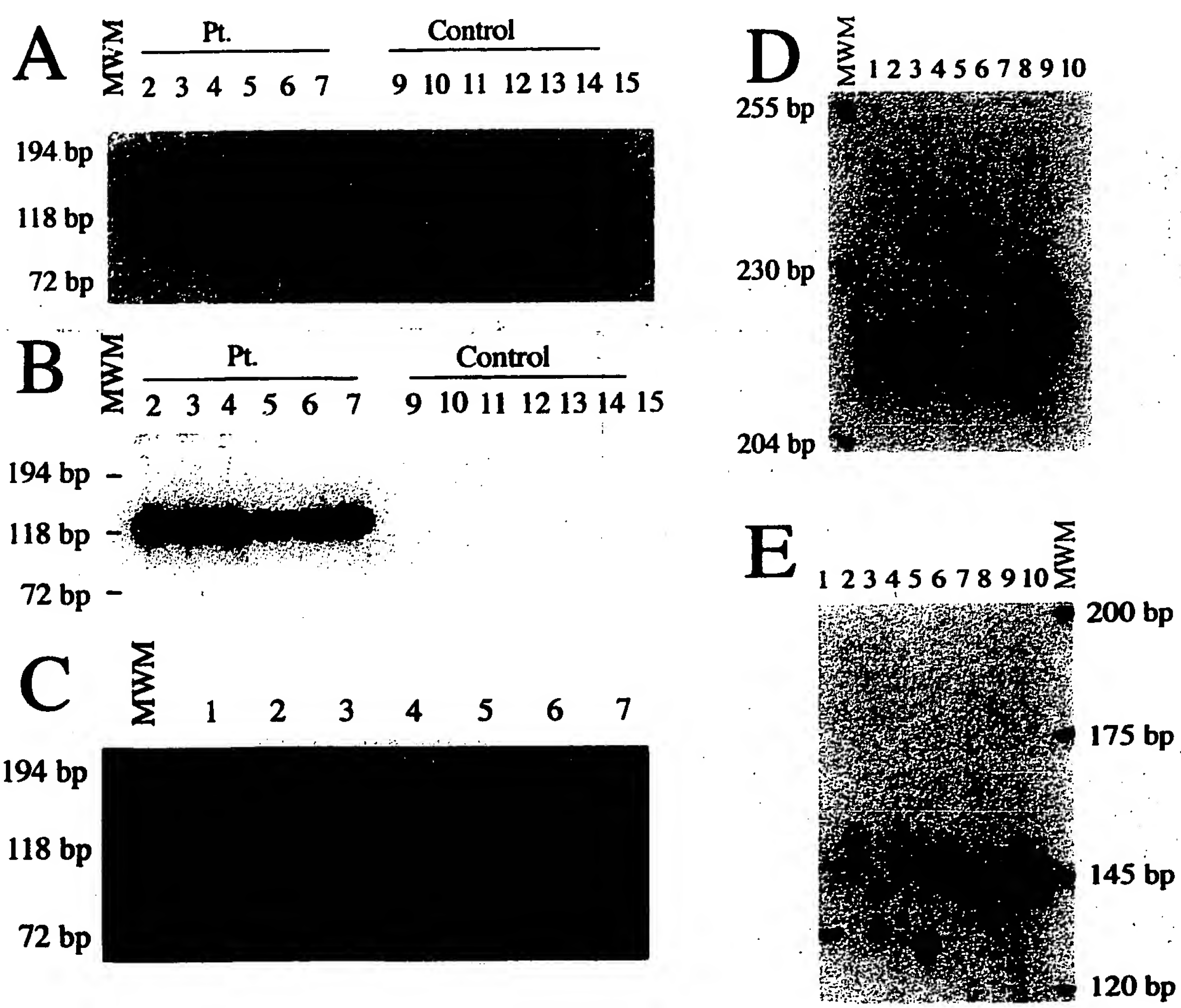

Figure 2. DNA from Epstein-Barr virus in lupus cases and matched controls. A concordant pair where both the case (*A*, lanes *2–7*) and its matched control (lanes *9–14*) have Epstein-Barr virus DNA in every PCR reaction tested. *B* presents a discordant pair where the lupus case has Epstein-Barr virus DNA (lanes *2–7*), while its matched control does not (lanes *9–14*). Lane *15* is a control with no added DNA. The products of the PCR using primers from the 60-kD Ro gene (nucleotide nos. 453–472 and 584–565) demonstrate that the PCR conditions will expand DNA in the control subjects who have apparently not been infected by Epstein-Barr virus. *D* and *E* present the polymorphisms at D1S1589 and D6S1027 found in the unique DNA from all controls with no Epstein-Barr virus DNA.

Table III. Detection of DNA from Epstein-Barr Virus in Cases of Pediatric Lupus and their Matched Controls

Cases	Controls	Number
Present	Present	23
Present	Absent	9
Absent	Present	0*
Absent	Absent	0

The presence and absence of Epstein-Barr virus DNA in 32 matched sets of lupus patients and controls support an increased frequency of Epstein-Barr virus infection in pediatric lupus. *$P < 0.002$, binomial test.

was retested, and had become Epstein-Barr virus–seropositive during the interval between specimens. The second subject was not available for retesting. Consequently, the Epstein-Barr virus DNA assay and Epstein-Barr virus seroconversion assay support the same conclusion: that there is a very close association with Epstein-Barr virus infection and lupus. Indeed, if the difference in the frequency of Epstein-Barr virus infection between controls and lupus patients is because Epstein-Barr virus causes lupus, then from the data in Table I the fraction of lupus cases attributable to Epstein-Barr virus infection is 98% (CI-95%, 85–99.7%).

Discussion

An association of lupus with Epstein-Barr virus infection has been found. Considered alone, the observed association of lupus with Epstein-Barr virus infection is consistent with three origins: a susceptibility for lupus in Epstein-Barr virus–infected persons, a susceptibility for Epstein-Barr virus infection in patients who previously developed lupus, or a third factor that causes independent susceptibilities to both Epstein-Barr virus infection and to lupus. At this time there is no evidence for such a third factor in this situation, but its existence must remain a formal possibility.

There are no data available to support an unusual and extreme susceptibility to Epstein-Barr virus in young lupus patients, and no mechanism for such a susceptibility is now known. Nevertheless, lupus is characterized by abnormally activated B cells, and a subset of B cells are the target for and site of Epstein-Barr virus infection. Perhaps the intrinsic resistance to Epstein-Barr virus infection is much lower in lupus patients because of the immunoregulatory changes that occur as a consequence of the disease. This possibility could only be eliminated from consideration by the demonstration that Epstein-Barr virus infection almost always precedes the onset of lupus. Unfortunately, no data exist with which to decide this issue. Consequently, a susceptibility to Epstein-Barr virus infection in lupus patients remains a plausible explanation for the observed association.

Fortunately, we are not restricted to considering the association of Epstein-Barr virus infection with lupus in the absence of insights from other work on Epstein-Barr virus or on lupus. Once the influence of confounding technical issues is removed, there is much consistent with the possibility that lupus arises in individuals who have been previously infected with Epstein-Barr virus, and is no observation that eliminates this possibility from consideration. Indeed, the experiments herein establishing association between Epstein-Barr virus and lupus were attempted as a test of the hypothesis that Epstein-Barr virus is an etiologic factor in the development of lupus. This hypothesis arose from immunochemical studies that suggested some of the critical features for a molecular mechanism, beginning with a structure from Epstein-Barr virus and culminating in one of the forms of lupus autoimmunity (2, 3).

The antigenic relationships between peptides from EBNA-1 and Sm B/B′ (2, 3) have suggested a possible mechanism for generating antispliceosomal autoimmunity, and may partially describe one of the ways in which Epstein-Barr virus–infected individuals can be predisposed to lupus (Fig. 3). First, Epstein-Barr virus is postulated to infect a susceptible person who is otherwise predisposed by genetics and environment to develop lupus. While virtually all people infected with Epstein-Barr virus develop anti–EBV-VCA antibodies, many also generate antibodies to other viral constituents, including EBNA-1. Among these, it is postulated that a few people may eventually develop antibodies to the PPPGRRP epitope on EBNA-1 which cross-reacts with the PPPGMRPP epitope on the spliceosome. The spliceosome is then processed as an antigen, and is presented to the immune system in a way that has not oc-

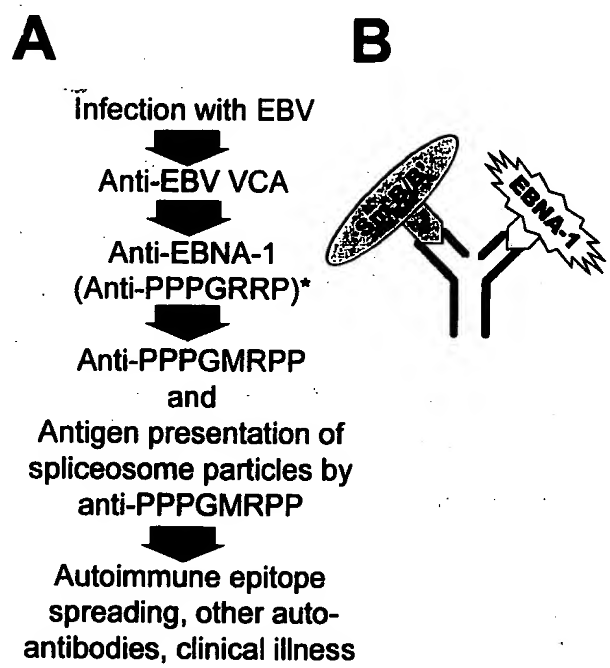

Figure 3. Hypothetical mechanism for association of Epstein-Barr viral infection and development of systemic lupus erythematosus. (*A*) People genetically susceptible to SLE may mount an unusual immune response (*) to EBNA-1. In one scenario humoral immunity against the PPPGRRP sequence may be a required step (2, 3). (*B*) These antibodies that react with PPPGRRP and cross-react with the PPPGMRPP as found in the native Sm B/B′ may initiate the cascade of autoimmune responses that spread to different regions of the spliceosomal autoantigens, and result in other autoimmune manifestations, including clinical disease. (*B* shows IgG binding to both Sm B/B′ and EBNA-1.)

curred previously, and against which tolerance has either not developed (immune ignorance) or is relatively weak. Lupus autoimmunity and clinical illness then result. There is no requirement in this model for either Epstein-Barr virus or any of the antibodies directed against it to be found in immune complexes, or to be deposited at sites of injury.

The cross-reaction of antibodies capable of binding both PPPGRRP as found in native EBNA-1 and PPPGMRPP as found in the native spliceosome (Fig. 3 *B*) may be a critical step allowing lupus autoimmunity to develop for some individuals (Fig. 3 *A*). Under this scenario we immunized animals with the selected peptides as previously reported (2, 3). This immunization with peptide bypasses the very complicated immune response against the chronic viral infection out of which lupus autoimmunity arises in the natural state. The peptide immunization directly induces a vigorous immune response against a structure that is capable of setting the immune system on a course toward lupus autoimmunity (2, 3). Not only are the anticipated cross-reacting antibodies observed, but also other characteristic features of lupus-like autoimmunity occur in the susceptible animals.

Of course, many steps in this hypothetical scenario for initiating lupus remain to be established. As emphasized above and at the most basic level, if this sequence of events is true, then Epstein-Barr virus infection must precede the onset of lupus. This prediction has not been tested. In addition, whether or not the many humoral autoimmune manifestations of lupus, such as antiribosomal P, anti-Ro (or SS-A), anti-La (or SS-B), or even anti-nRNP, develop by analogous molecular mimicry mechanisms, must necessarily be the subject of other work. Perhaps the binding of La to the EBER RNAs encoded by Epstein-Barr virus and expressed in some infected cells (14) provides an alternative mechanism.

If one accepts that lupus is essentially a humoral autoimmune disease, then another prediction of the hypothesis that Epstein-Barr virus causes lupus is that the immune response against the virus must have critical distinguishing characteristics that would allow lupus autoimmunity to be initiated. At most, we could consider present knowledge to provide only suggestive clues for what these critical distinguishing characteristics might be. On the other hand, there are many differences between lupus patients and normal controls in their immune responses to Epstein-Barr virus, consistent with the existence of lupus autoimmunity-inducing differences in the immune response.

For example, when comparing lupus sera to control sera, several investigators have detected increases in the level or differences in the fine specificity of anti–early antigen, antimembrane antigen, anti-EBNA-1, EBNA-2, or EBNA-3 (15–20). In two studies these findings do not appear to be explained by polyclonal B cell activation, since titers to other antigens, measured concurrently, were not elevated (18, 20). In addition, the only published study systematically examining the immune response to EBNA-1 peptides outside of the glycine–alanine repeat showed several significant differences between adult lupus patients and normal controls (18). Of the 11 different peptides studied, 3 of these peptides were commonly bound by lupus patient sera at a higher level than that found in normal control sera. In addition, the increased binding to PPPGRRP (from EBNA-1) by lupus sera containing anti-Sm and anti-nRNP has also been appreciated (21). Recent preliminary studies from our laboratory with over 400 overlapping octapeptides spanning the EBNA-1 protein show that pediatric lupus patient sera consistently bind different regions of EBNA-1 than do their matched, anti–EBV-VCA–positive controls (J.A. James and J.B. Harley, unpublished data). Among these differences is the tendency for normal sera to bind the glycine–alanine repeat of EBNA-1 while lupus sera do not generally bind this amino acid repeat. In contrast, a glycine–arginine repeat that is found in both EBNA-1 and Sm D is more antigenic for lupus sera than it is in control sera (22–26). These findings are consistent with the possibility that there may be multiple antigenic relationships between lupus and Epstein-Barr virus, and that critical ones vary among lupus patients.

Other features that may allow or encourage a role for Epstein-Barr virus in lupus include virus latency (4) and deficiency in the T cell immune suppression required to contain this infection. In a study by Tsokos et al. (27), T cells of SLE patients have been shown to be deficient in their ability to suppress the outgrowth of Epstein-Barr virus–infected B cells from in vitro–infected peripheral blood cells. Latency and the immune response against the virus are different aspects of the same process; the virus has many molecular features that appear to encourage latency and discourage an infection-terminating immune response. The glycine–alanine repeat appears to be responsible for the described defects in antigen processing of EBNA-1 (28). Indeed, normal individuals do not mount cytotoxic T cell responses against EBNA-1 (28). Apparently, the glycine–alanine repeat of EBNA-1 appears to inhibit HLA class I peptide presentation from EBNA-1, thereby inhibiting T cell immunity against this antigen (28). This incompletely understood effect may profoundly influence the immune response, though its importance to the pathogenesis of lupus is not known.

Epstein-Barr virus has an IL-10–like gene that has many similar and some different activities relative to human IL-10 (29). For example, both human and viral IL-10 inhibit apoptosis in T cells recovered from patients with infectious mononucleosis (30). Clearly, viral IL-10 has the potential to influence the immune response to the virus (31, 32).

Epstein-Barr virus also infects B cells, which then become dysregulated and proliferate. Indeed, apoptosis of B cells is abnormal as a consequence of Epstein-Barr virus infection which, thereby, has the potential to favor development of autoimmunity (33). Epstein-Barr virus can increase B cell survival of latently infected cells both by upregulating cellular *bcl-2* and through an Epstein-Barr virus–encoded protein, BHRF1. BHRF1 has amino acid sequence similarity and a molecular action similar to *bcl-2* in that this gene inhibits apoptosis in B cells (34). Interestingly, when transgenic mice are stimulated to overproduce *bcl-2* they develop a syndrome of lupus-like autoimmunity and immune complex–mediated, cresentic glomerulonephritis (35). Epstein-Barr virus infection is also well known to induce the production of autoantibodies during acute infection (25, 36).

When the association between Epstein-Barr virus and lupus described herein is considered in the context of other studies, the aggregate of what is known is consistent with the intriguing possibility that Epstein-Barr virus infection is required, but not alone sufficient, for development of systemic lupus erythematosus in most patients. We look forward to the results of those subsequent experiments that will support and establish or disprove this hypothesis.

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virus-related cross reactive autoimmune response in multiple sclerosis in Norway

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AB In studies of patients in Norway with multiple sclerosis (MS), we have found cross reactive autoantibodies related to the **Epstein Barr** virus nuclear antigen-1 (EBNA-1). The MS patients had elevated IgG antibody to EBNA-1, as measured by reactivity with a synthetic glycine/alanine peptide, P62, which represents the glycine/alanine repeat in EBNA-1. The mean titer of anti-P62 in patients with acute relapse at the time of **assay** was significantly higher than in the remaining patients. Patients with remitting/relapsing MS also had elevated autoantibody to a lymphocyte protein, p542, cross reactive with EBNA-1 through a glycine/serine epitope. High titered anti-EBNA-1 antibodies from some MS, as well as from some **SLE** sera, were shown to cross react with 80-82 kDa and 60 kDa proteins in neuroglial cells.

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An Epstein Barr virus-related cross reactive autoimmune response in multiple sclerosis in Norway

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Abstract

In studies of patients in Norway with multiple sclerosis (MS), we have found cross reactive autoantibodies related to the Epstein Barr virus nuclear antigen-1 (EBNA-1). The MS patients had elevated IgG antibody to EBNA-1, as measured by reactivity with a synthetic glycine/alanine peptide, P62, which represents the glycine/alanine repeat in EBNA-1. The mean titer of anti-P62 in patients with acute relapse at the time of assay was significantly higher than in the remaining patients. Patients with remitting/relapsing MS also had elevated autoantibody to a lymphocyte protein, p542, cross reactive with EBNA-1 through a glycine/serine epitope. High titered anti-EBNA-1 antibodies from some MS, as well as from some SLE sera, were shown to cross react with 80–82 kDa and 60 kDa proteins in neuroglial cells.

Keywords: Multiple sclerosis; Epstein Barr virus; Autoantibody; Epstein Barr virus nuclear antigen-1 (EBNA-1); Neuroglial

1. Introduction

The possibility that the Epstein Barr virus (EBV) may play some role in the etiology or pathogenesis of multiple sclerosis (MS) has been suggested by both immunological and epidemiological observations. Increased levels of antibody to EBV viral capsid antigen and to the EB nuclear antigen-1 (EBNA-1) have been noted in MS sera and spinal fluids (Sumaya et al., 1980; Bray et al., 1983, 1992a,b; Larsen et al., 1985), and a deficiency in T cell ability to suppress the in vitro growth of autologous EBV-infected B cells from MS patients has been documented (Craig et al., 1988). In a retrospective epidemiological evaluation of the past histories of 941 MS patients, the patients were asked to recall whether they had had any of nine previous illnesses varying from measles to polio. Only infectious mononucleosis was recalled by the patients significantly more often than by their matched controls, 13% versus 2% (Operskalski et al., 1989). Martyn et al. (1993) confirmed this observation in a separate survey, with frequencies found similar to those reported by Operskalski et al. (1989). In a more prospective approach, Lindberg et al. used the registries for infectious mononucleosis and MS in Goteborg, Sweden, and found more MS (3 cases) following documented infectious mononucleosis than in matched controls (none), a slight but statistically significant difference (Lindberg et al., 1991).

In a Norwegian population, Riise et al. (1991) observed that MS patients with the same birth cohort had lived significantly closer to each other than would be expected during ages 13–20 years, with peak clustering at age 18. The present report is of follow-up observations on this Norwegian study and relates to the possibility that the observed ages implicate infection by EBV. In independent studies in La Jolla, we had observed that (1) acute infection by EBV induces numerous autoantibodies cross reactive with the EBNA-1 molecule through EBNA-1's long glycine/alanine repeat, a region in the molecule that con-

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stitutes its principal antigenic site (Rhodes et al., 1987; Rumpold et al., 1987), and (2) that a recombinant expression product of one of the B lymphocyte autoantigens (p542) has a 28-mer, GGGASGGGGGGGGSGGGGSGG-GGGGGSS, which is the site on this autoantigen that is cross reactive with EBNA-1 (Vaughan et al., 1995b). In the present study, we wished to see whether an EBV-related autoimmune response also is expressed in the Norwegian MS patients.

We report here that anti-EBNA-1 titers, as measured by an anti-peptide assay, were elevated in the MS patients' sera, especially during exacerbations in patients with remitting/relapsing disease (R/R MS); that anti-p542 autoantibodies also were elevated in R/R MS, and were EBV-related as judged by affinity purification and inhibition studies; and that anti-EBNA-1 antibodies from sera with high titers of anti-EBNA-1 also frequently exhibit cross reactivity with 80–82 kDa or 60 kDa antigens present in neuroglial cell lines. Although these antibodies are not disease specific, the possibility that they may play an adjunct role in pathogenesis should be considered.

2. Materials and methods

2.1. Sera

The sera evaluated in this study were from the series reported by Riise et al. (1991). A random sample of 36 MS patients and 36 controls were selected out of a total case-control material of 155 patients and 200 age-, area-, and sex-matched hospital controls. This material, which is described in detail by Gronning et al. (1993), consisted of all MS patients who had their onset of disease between 1976 and 1986 in Hordaland County, Norway.

The mean ages at time of examination (1987) was 36.7 years (range 18–54 years) for both the cases and controls included in the present study; 58% of cases and 56% of controls were female. All MS patients were examined in 1989 by a neurologist using the criteria of Bauer (Bauer, 1980); twenty nine of the 36 patients (80%) selected for the present study were classified as definite, six (17%) as probable, and one (3%) as possible MS. Upon re-examination in 1995, all but one was classified as definite. The patients were also classified according to the clinical course; thirteen (36%) had a remitting course, 17 (47%) a remitting with a secondary progressive course, and 6 patients (17%) a primary progressive course. A total of six patients (17%) with remitting disease were having an acute clinical relapse at the time their blood samples were taken.

Eighteen Norway controls were hospitalized patients on the neurological service. Two had migraine, 14 lumbar disc syndromes, and 2 (K023 and K024) rheumatoid arthritis. For K023 there was also suspicion of MS. The remainder of the 18 controls were from the ear/nose/throat, plastic surgery, and gynecology departments, and in none was MS or rheumatic disease a diagnostic suspicion.

The sera from patients with systemic lupus erythematosus, and those from La Jolla and San Diego normal volunteers, have been described previously (Vaughan et al., 1995a).

2.2. Antigens, autoantigens, and mutants

The characteristics of p542 and of its isolated and purified recombinant protein have been described (Vaughan et al., 1995b,a). The protein was generated as a hexahistidine fusion protein suitable for affinity purification on a nickel column.

Deletion mutants of p542 (Vaughan et al., 1995a) were generated from unique restriction sites on either side of the glycine rich 28-mer (Fig. 1). Cuts were made at *Eco*NI and *Hin*dIII (downstream in the polycloning site of the plasmid), *Cla*I and *Eco*NI, and *Cla*I and *Hin*dIII to generate D1, D2, and D3 mutants, respectively. For D2, re-ligation was in frame. The mutants were of the expected sizes in polyacrylamide electrophoresis.

Recombinant EBNA-1 was expressed in transfected COS-7 monkey kidney cells (American Type Culture Collection, Rockville, MD) in 100 mm petri dishes, as de-

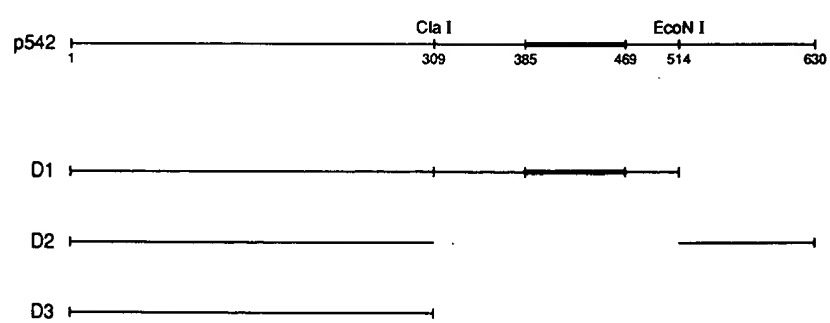

Fig. 1. Schematic of the p542 gene fragment and the deletion mutants constructed from it. The p542 DNA was cut at the *Cla*I or *Eco*NI restriction site (nucleotides 309 or 514, respectively), and at a *Hin*dIII site downstream in the plasmid, with blunt ending and re-ligation to generate DNA encoding the D1 and D3 mutant proteins. D2 was generated from the DNA cut simultaneously by *Cla*I and *Eco*NI, blunt ending and re-ligation, which occurred in frame. The DNA encoding the glycine rich sequence, 385–469, is indicated in heavy line.

scribed previously (Vaughan et al., 1995b). The cells were lysed and the proteins separated in polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western blotting. The EBNA-1 was displayed as an 80 kDa band not seen in non-transfected COS-7 cells, and could be used in this form to detect anti-EBNA-1 activity. Type II human collagen was the gift of Dr. Steffen Gay, University of Alabama Medical School. Actin, insulin, and bovine thyroglobulin were purchased from Sigma Chemical, St. Louis, MO. Pneumococcal vaccine (Pneumovax) and tetanus toxoid were the licensed immunization products of Merck, Sharp and Dohme, Rahway, NJ. The heat shock protein groEL was a recombinant product and the gift of Dr. David Yu, University of California, Los Angeles. The human heat shock protein huHsp60-H is the hexahistidine fusion protein of huHsp60 prepared in this laboratory from the huHsp60 (P1) gene provided to us by Dr. Radhey Gupta, McMaster University, Hamilton, Ontario. Influenza vaccine was from Wyeth Laboratories, Marietta, PA.

2.3. Immunoaffinity purification of anti-p542

Solid phase p542 autoantigen was prepared for immunoabsorption as follows. Bacterial extracts containing the recombinant protein were electrophoresed in polyacrylamide gels and transferred to nitrocellulose strips. The portion of the strips containing the autoantigen bands were cut out, washed, dried, and reacted with sera known to have anti-p542 activity. Antibody was eluted from the autoantigen at pH 11.5 and then promptly neutralized, as described (Vaughan et al., 1995b). Equal-sized portions from other areas of the blot were similarly treated and used as negative controls.

2.4. Enzyme-linked antibody assays (ELISA)

Assays for autoantibodies were carried out in 96-well microtiter trays (Costar), as previously described (Vaughan et al., 1995b). After preliminary assays to determine the optimal concentration of antigen, the wells were coated with antigen at 10 μg/ml for 1 h at 37°C, the wells blocked with 1% bovine serum albumin, and the antibody in PM applied for 1 h at room temperature. Sera being examined for autoantibody to p542 were blocked, additionally, with extracts of *Escherichia coli* infected by phage carrying antisense inserts. All sera were examined at 1:100 dilutions, except sera assayed for anti-pneumococcus and anti-influenza, which were examined at 1:1000 and 1:10,000, respectively. These dilutions were chosen after preliminary studies determined that each was on the downslope of the titration curve for its respective antigen, and that the dilution was therefore appropriate for assaying for relative antibody contents between groups of sera. The detecting antibody was rabbit anti-human IgG (Boehringer Mannheim, Indianapolis) conjugated with horse radish peroxidase.

3. Results

3.1. Anti-p542 reactivity and specificity in MS sera

The mean level of IgG anti-p542 for the 35 sera from patients with definite MS (cf. Materials and Methods) was 0.521 ± 0.088 O.D., compared to 0.267 ± 0.066 O.D. for the controls (Table 1 and Fig. 2). This difference increased when patients with chronic progressive MS (mean anti-p542 of 0.220 ± 0.094 O.D.) were removed from the group, an adjustment suggested by the reports by others that chronic progressive MS and R/R MS may differ from each other not only immunologically, but also by genetic markers, magnetic resonance imaging, and neurological manifestations (Warren et al., 1994; Stevens et al., 1992; Olerup et al., 1989; Thompson et al., 1991). The anti-p542 values in our MS patients did not correlate significantly with sex, age of onset, duration, or activity of disease.

To investigate the specificity of the anti-p542, we purified the autoantibody from the sera of 3 R/R MS patients by immunoaffinity using solid phase p542 (as the β-galactosidase fusion protein) and tested the reactivities of these preparations in ELISA against p542 as a hexahistidine fusion protein, and against a variety of other proteins (Fig. 3). The anti-p542 was very specific: while it reacted very strongly with the recombinant p542/hexahistidine fusion protein, it did not react with any of the unrelated proteins or peptides tested. Nor did it react with two other recombinant proteins, huHsp60-H and p554-H (Vaughan et al., 1995b), which had the same polyhistidine tail carried by p542. In all these respects, the anti-p542 from these three MS sera were quite similar to affinity purified anti-p542 from SLE sera, as we have reported elsewhere (Vaughan et al., 1995a).

To show that purified anti-p542 antibodies are indeed related to EBNA-1, we tested their abilities to react in Western blots with natural EBNA-1 in the WiL2 line of B lymphocytes, and with recombinant EBNA-1 molecule in

Table 1
IgG antibody titers in multiple sclerosis and control sera

	Anti-P62			Anti-p542		
	all MS	R/R MS [a]	control [b]	all MS	R/R MS [a]	control [b]
No.	35	29	36	35	29	36
Mean	1.633	1.661	1.156	0.521	0.562	0.267
SEM	0.078	0.090	0.106	0.088	0.102	0.066
P-value [c]	0.0006	0.0008			0.033	0.014
P-value [d]	0.0001	0.0001			0.055	0.025

Sera were examined at 1:100 dilutions in ELISA in triplicate, and the antigens were used at 10 μg/ml to coat the wells.
[a] Patients with relapsing/remitting disease.
[b] 36 hospitalized non-MS patients in Norway.
[c] 2-tail *P* by unpaired 2 group *t*-test using StatView 512+ Statistics Utility (Brain Power, Calabasas, CA), comparing to the controls.
[d] Mann Whitney rank sum test, using StatView 512+, comparing to the controls.

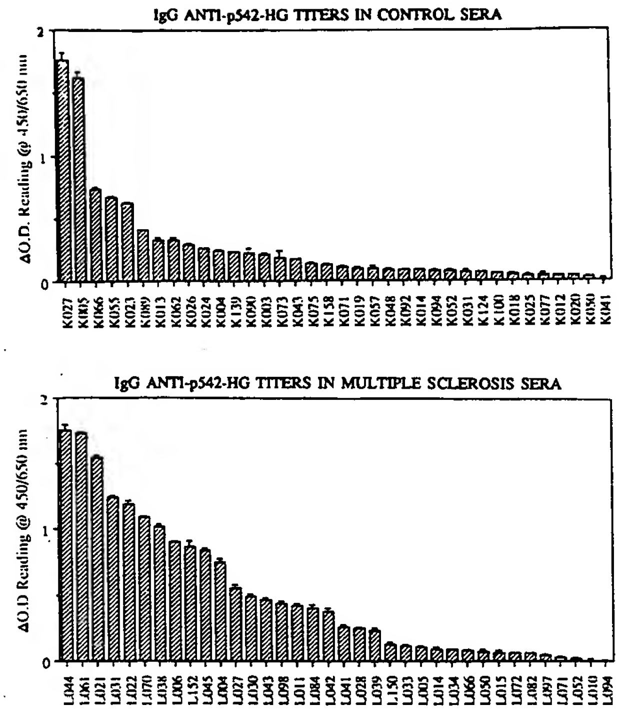

Fig. 2. IgG anti-p542 titers by ELISA on patients with multiple sclerosis (below) and their age- and sex-matched hospital controls (above) from the series studied by Riise et al. (1991). The designation p542-HG specifies that an additional gel separation step has been added during the purification of p542-H (the hexahistidine fusion protein of p542).

monkey kidney cells. The anti-p542 preparations reacted with the EBNA-1 in both cell types. The reactions with the recombinant EBNA-1 are shown in Fig. 4. This reactivity was specifically inhibited by pre-incubation of the autoantibodies in 10 μg/ml of the peptide P62, confirming that the reactions were with the glycine/alanine repeat in EBNA-1 (not shown).

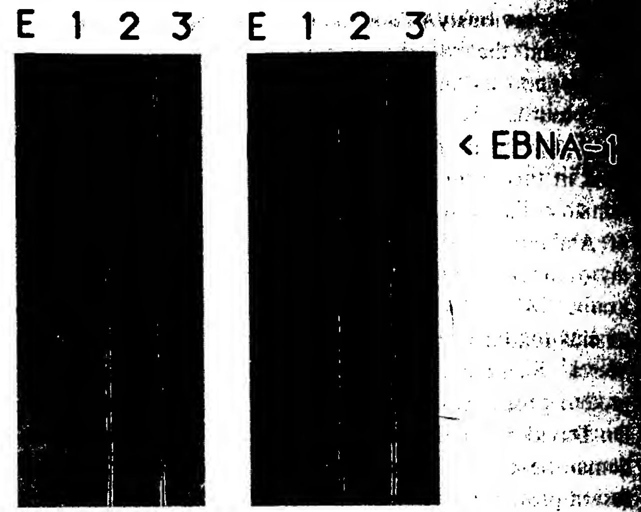

Fig. 4. Reactivity in Western blots of immunoaffinity purified anti-p542 with recombinant EBNA-1. In the right panel, an extract of monkey kidney cells expressing EBNA-1 has been electrophoresed through poly-acrylamide gel, transferred to nitrocellulose strips, and the strips probed with known antibody to EBNA-1 (lane E) or with the three anti-p542 preparations described in Fig. 3 (lanes 1, 2, and 3). In the left panel, the same procedure has been carried out, except that the monkey kidney cells had not been transfected with EBNA-1 DNA.

The reactivities of the anti-p542 positive sera were then examined with deletion mutants of p542, to determine whether the primary reactivities would be, as predicted, to D1 (Fig. 1), the mutant containing the cross reacting glycine rich 28-mer. The results are shown in Fig. 5. Anti-D1 reactivity was predominant in almost all cases and exclusive in some. A lesser degree of anti-D2 reactivity was seen in some sera; anti-D3 reactivity was seen in one. The anti-D2 and anti-D3 reactivities suggest secondary epitope spread, as discussed elsewhere (Vaughan et al., 1995a).

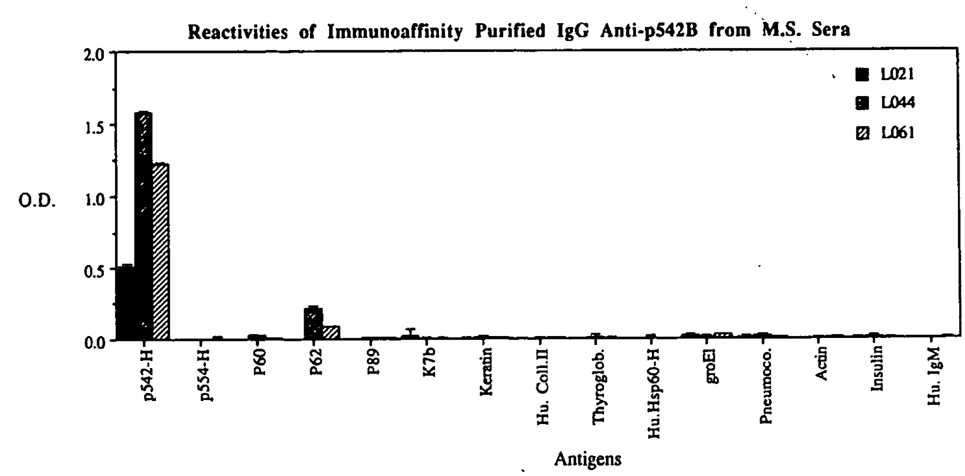

Fig. 3. Specificity of IgG anti-p542 in three MS sera. The anti-p542 from each of three MS sera was immunoaffinity purified from solid phase recombinant p542-B (the β-galactosidase fusion protein) and reacted in ELISA with p542-H (the hexahistidine fusion protein), a system assuring that no conceivable antibody reactivity with the fusion products can enter into the results. Other proteins and peptides tested: p554-H is the fusion protein of a second autoantigen seen in infectious mononucleosis (Vaughan et al., 1995b); P60 and P62 are glycine/alanine peptides present in EBNA-1; P89 and K7b are non-glycine/alanine peptides seen, respectively, in EBNA-1 and in EBV early antigen (EA-R); pneumoco. is the pneumovax preparation; and the remaining proteins are purified preparations of each. All antigens and peptides were used at 10 μg/ml to coat the plates.

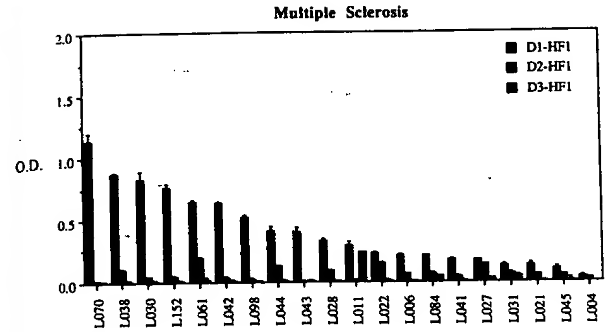

Fig. 5. Reactivities in ELISA of the MS sera with deletion mutants of p542 (see Fig. 1). The sera examined are the twenty that had been most reactive with p542 in Fig. 2. The antigens were used at 5 μg/ml to coat the plates, and the sera were examined at 1:100.

3.2. Relation of anti-p542 to anti-P62

P62 is a peptide, AGAGGGAGGAGAGGGAGGAG, which is present in the glycine/alanine repeat of EBNA-1. A major portion of antibody to EBNA-1 reacts with this peptide (Rumpold et al., 1987). We measured anti-P62 levels in the MS and control sera. The mean titer of IgG anti-P62 of the patients was significantly greater than that of the controls (Table 1), consistent with the prior reports of Bray et al. (1983, 1992b) of elevated anti-EBNA-1 titers in MS. This difference from the controls was present in titration studies out to dilutions of 1:6400 (not shown). We then compared the anti-p542 reactivities with anti-P62 reactivities in the MS sera (Fig. 6). The highest titers of anti-p542 occurred in the sera with the highest anti-P62 titers. Although this relationship does not reach statistical significance, the grouping suggests that individuals having the strongest anti-P62 (anti-EBNA-1) immune responses were those most liable to development of the cross reacting anti-p542.

3.3. An EBNA-1-related anti-neuroglial autoantibody

To look for other possible cross reactions of anti-EBNA-1 in MS and control sera, we purified anti-EBNA-1

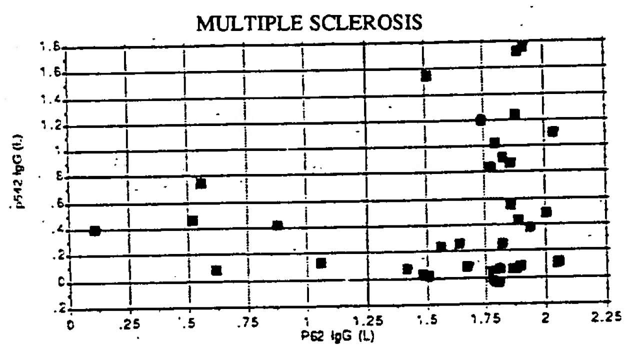

Fig. 6. Relation of IgG anti-P62 peptide levels to IgG anti-p542 autoantibody levels in patients and controls. The tendency of the sera with the higher anti-p542 levels to also have higher anti-P62 levels is not statistically significant, but the grouping prompted experiments to test sera with high titers of anti-P62 for other cross reactive autoantibodies (see text).

Table 2
Anti-82 kDa reactivity of immunoaffinity purified anti-EBNA-1 from various sera with C6 neuroglial cells

	Anti-P62 reactivity in serum (O.D.)		
	0.2–0.6	1.6–1.8	> 1.9
MS	0/5 [a]	0/1	3/8
SLE	0/5	2/4	1/4
Controls	0/5	2/8	– [b]

Antibody assays were carried out as described in Table 1.
[a] Positive preparations/number of preparations tested.
[b] None available.

antibody from selected sera with high anti-P62 (≥ 1.6 O.D. by ELISA, a level displayed by half of the MS patients, but by only 15–20% of normals or SLE patients (Vaughan et al., 1995a)) or low anti-P62 (0.2–0.6 O.D.) and tested them in Western blots against mouse brain or C6 (ATCC CCL 107) murine neuroglial cell extracts. The immunoaffinity purification was carried out with solid phase recombinant EBNA-1 from monkey kidney cells (see Materials and Methods). Purified anti-EBNA-1 reacted with an 82 kDa antigen in both mouse brain and the C6 neuroglial cells (Table 2). No sera with low titers of anti-P62 yielded preparations that were reactive with the 82 kDa protein. Of the 9 MS sera, 8 SLE sera, and 8 control sera which had high anti-P62, 3, 3, and 2, respectively, yielded antibody with anti-82 kDa reactivity. Thus the development of anti-82 kDa is more related to the amount of anti-glycine/alanine (EBNA-1) antibody than to a specific disease.

The anti-82 kDa reactivities could not be related to anti-p542 activity, since two of the 8 anti-82 kDa positive preparations illustrated in Table 2 came from sera lacking anti-p542. They were, however, related to EBNA-1 through EBNA-1's glycine/alanine repeat, as shown in inhibition studies (Fig. 7). The anti-EBNA-1 preparations from three different sera, used as probes against C6 cell extracts, were completely inhibited by pre incubation of the antibodies with 10 μg/ml P62.

To investigate whether anti-EBNA-1 antibodies from these sera would cross react also with antigens in human neuroglial cells, we employed the H4 cell line (ATCC HTB 148) derived from a patient with a neuroglioma. Here the antibodies reacted with an 80–82 kDa doublet and a 60 kDa singlet band (Fig. 8). These antibody reactivities were inhibited by P62 at 10 μg/ml (not shown).

3.4. Anti-P62 in relation to disease activity

We have made the further observation that the mean titers of anti-P62 in the six patients who were in acute clinical relapse at the time of examination (see Materials and Methods) were significantly higher than were those of the patients in stable phases of their disease, whether calculated on the basis of the 29 patients remaining from the total series of 35 patients, or on the basis only of the 23

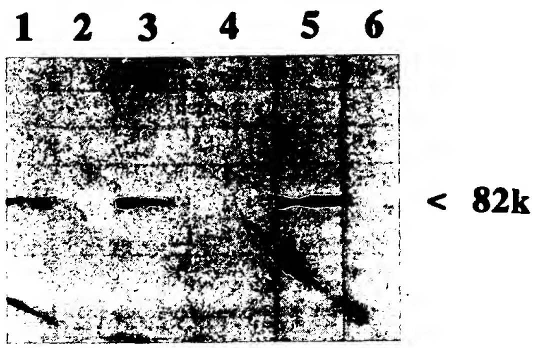

Fig. 7. Inhibition of the reactivities of immunoaffinity purified anti-EBNA-1. One of each of the positive anti-EBNA-1 preparations from the MS, SLE, and normal groups in Table 3 were used as probes in Western blots against C6 cell extracts. In lanes 1, 3, and 5 the respective autoantibodies were pre-incubated with 10 μg/ml of a non-glycine/alanine EBNA-1 peptide ($E_{611-625}$); in lanes 2, 4, and 6 the autoantibodies were pre-incubated with 10 μg/ml of the glycine/alanine EBNA-1 peptide, P62. The single 82 kDa band seen by the autoantibody preparations was completely inhibited in each case by the P62. The diagonal marks are artifacts from forceps handling of the strips.

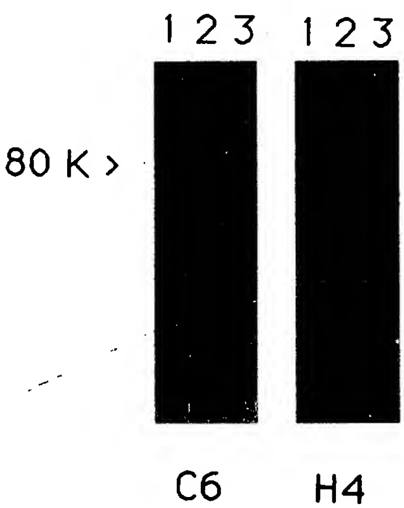

Fig. 8. Comparison of the reactivities of immunoaffinity purified anti-EBNA-1 with neuroglial cell proteins in murine (C6) and human (H4) neuroglial cell extracts. The Western blots were carried out in parallel with each other. The preparations of anti-EBNA-1 were from 2 MS sera (lanes 1 and 2) and an SLE serum (lane 3).

Table 3
IgG anti-P62 titers in MS patients

	In relapse	Stable	
	R/R MS	all MS	R/R MS
No. of patients	6	29	23
Mean	1.90	1.58	1.69
Std. error	0.053	0.091	0.109
P-value [a]		0.0041	0.018
P-value [b]		0.026	0.059

Antibody assays, patients, and comparisons were as described in Table 1.
[a] T-test, separate variance estimates.
[b] Mann Whitney rank sum test.

Table 4
Relative quantities of irrelevant antibodies in MS and Norway controls sera

	MS	Controls	P-value [a]
Pneumo	1.904 ± 0.512 [b]	1.743 ± 0.539	0.197
huHsp 60	0.071 ± 0.075	0.107 ± 0.114	0.110
Influenza	1.068 ± 0.560	1.169 ± 0.438	0.397

Assays for antibodies as described in Table 1, except that the serum dilutions examined for pneumo and influenza were 1:1000 and 1:10,000, respectively.
[a] Comparisons between MS and controls.
[b] Mean ± SD.

remaining patients with remitting/relapsing disease (Table 3). Only three of these 6 sera were still available later in our studies when all sera were titered to 1:6400, because the six had been among those used for the specificity and cross reactivity studies described above. The means for the anti-P62 of the remaining three were 2.32, 1.86, and 0.78 O.D. at 1:400, 1:1600, and 1:6400 dilutions, respectively, as compared with 1.76, 1.26, and 0.054 O.D. for the means of the 29 sera of the patients not in clinical relapse. In contrast to anti-P62, anti-p542 titers were not higher in the sera of the six patients in acute relapse.

3.5. *Lack of evidence for a generalized polyclonal B cell activation in MS*

It could be argued that the anti-P62, anti-p542, and the anti-80/82 kDa autoantibody responses we have documented here are simply manifestations of a generalized polyclonal B cell activation in MS. To investigate this possibility, we compared the antibody contents of the MS and control sera to pneumococcal, influenza, and huHsp60 autoantigen preparations (Table 4). For each antigen, the serum dilution examined was on the downslope of the respective titration curve. In none of the comparisons was there a significant difference between the mean antibody contents of the MS sera and those of the controls. Thus, not all antibodies are involved in the stimulatory process that invokes the anti-P62, anti-p542 and anti-80/82 kDa responses in MS.

4. Discussion

In this series of Norwegian patients with MS, we show an enhanced immune and autoimmune response related to the EBV antigen, EBNA-1. This is characterized by the following: (1) high antibody levels against the glycine/alanine peptide, P62, representing the long glycine/alanine repeat in the EBNA-1 molecule; (2) a significant association of high anti-P62 levels with bouts of activity in R/R disease; (3) high autoantibody titers in R/R disease to p542, an autoantigen which cross reacts with EBNA-1 through its glycine rich epitope; and (4) the occurrence in high titer sera of anti-EBNA-1 antibodies that cross react

with an 82 kDa antigen in murine neuroglial cells, and with 80–82 and 60 kDa antigens in human neuroglial cells, through EBNA-1's glycine/alanine repeat. While none of these antibodies is exclusively found in MS, they do document an exaggerated immune response to EBV in MS and an associated cross reaction with a cell type involved in the disease.

Multiple antibodies are well known to be elevated in MS (Sun, 1993; Norrby, 1978; Cremer et al., 1980; Forghani et al., 1980; Salmi et al., 1983; Shirodaria et al., 1987; Link et al., 1990), and the elevations we describe here of anti-P62 and anti-p542 add to the list. Some have thought that these elevations may be due to non-specific, generalized polyclonal B cell activation, and therefore perhaps of less interest. However, other antibodies present in the serum (cf. Table 4) are not elevated. Presumably, therefore, antigen-specific stimulation contributes to the elevations, and for anti-p542 EBNA-1 seems the likely antigen.

We show that anti-p542 is not polyreactive, that its predominant reactivity is with the deletion mutant of the protein that still has its glycine rich sequence, and that the cross reactivity with EBNA-1 is inhibitable with the glycine/alanine peptide, P62. The polyglycine sequence in p542 thus constitutes its cross reactive epitope. Similar glycine rich sequences are found in multiple proteins in eukaryotic cells, and also in some viruses. Anti-p542 thus is a prototype autoantibody reactive with what may be a new class of glycine rich epitopes. The two cross reactive neuroglial proteins we have identified may, as evidenced by their inhibition by P62, be part of such a class.

The cross reactions we have shown do not precisely incriminate EBV or EBNA-1 in generation of the autoantibodies, since glycine rich sequences similar to those in EBNA-1 and p542 are present also in other virus species (discussed in Vaughan et al., 1995b). So other viruses may be able to generate similar autoantibodies. We have noted this in CMV-induced infectious mononucleosis (Rhodes et al., 1990). However, the serological and epidemiological studies we noted in our introductory comments (Sumaya et al., 1980; Bray et al., 1983, 1992a,b; Larsen et al., 1985; Craig et al., 1988; Operskalski et al., 1989; Martyn et al., 1993; Lindberg et al., 1991; Riise et al., 1991) suggest that it would be EBV in MS. By a computer search of the GenEmbl and SWISS-PROT database libraries, neither measles nor any of the other viruses that have been considered as possible inciting agents in MS, have proteins containing glycine rich sequences. Among herpes viruses, CMV proteins do have such sequences, but Bray et al. (1983) did not find elevated anti-CMV titers in MS. By our computer search, human herpes virus-6 (HHV6) also does not have them. The possibility exists, nevertheless, that microbial antigens devoid of glycine rich sequences, but nevertheless having configurations capable of eliciting antibodies with cross reactions inhibitable by P62, could have elicited the autoantibodies we describe here.

Our initial studies were directed to the amounts and specificities of anti-p542 in MS sera, and these served to support our conclusion that there is an increased EBV-related autoimmune response in MS. We later recognized, however, that acute exacerbations of disease in patients with remitting/relapsing MS were associated with very high values of anti-P62, but not of anti-p542. If an EBNA-1-related autoimmunity were to have meaning in pathogenesis, therefore, it would presumably have to be through cross reactions other than that with p542. The cross reactions of anti-EBNA-1 with the 82–80 kDa and 60 kDa proteins in neuroglial cells might be considered for this.

Investigation of the distribution of the 80–82 kDa and 60 kDa proteins has shown that they are not limited to neuroglial cells, but that they also occur in T lymphocytes and possibly in smaller amounts in other cells (not shown). Additionally, neither anti-p542, as shown in Vaughan et al. (1995a), nor autoantibodies to the neuroglial proteins (Table 2), are exclusive for MS. Clearly, there is lack of tissue specificity, as well as of disease specificity, for these autoantibodies. Does this make them insignificant in respect to pathogenesis? We do not know the answer to this, but the possibility that an EBV-induced autoimmunity can, in presence of other more disease-specific and localizing influences, contribute important collaborative effects in pathogenesis seems appropriate for further investigation.

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anticoagulants in an individual with systemic lupus erythematosus

Nakamura, Norihiko; Azuma, Chihiro; Akamizu, Takashi; Sugawa, Hideo; Matsuda, Fumihiko; Mitsuda, Nobuaki; Honjo, Tasuku; Mori, Toru; Yamaji, Kenji

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AB From one patient with **systemic lupus** erythematosus retaining lupus anticoagulant (LAC), 6 **Epstein-Barr** virus-transformed human B cell clones secreting antibodies that affect the coagulation **assay** were established. Two and 4 of the clones secreted IgM and IgG antibodies, resp. Although all 6 antibodies displayed anticardiolipin activity in ELISA, the increased binding activity in the presence of .beta.2-glycoprotein I was limited only to the IgG antibodies. Five antibodies (two IgM and three IgG) had LAC activity which prolonged the activated partial thromboplastin time (APTT), whereas one IgG antibody shortened the APTT. Two of the IgG producing clones had an identical Ig heavy chain gene rearrangement despite their opposite effects on the coagulation **assay**. These results demonstrated the heterogeneity of LACs and diversity among their physiol. functions.

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HETEROGENEITY AND DIVERSITY OF IgM AND IgG LUPUS ANTICOAGULANTS IN AN INDIVIDUAL WITH SYSTEMIC LUPUS ERYTHEMATOSUS+

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From one patient with systemic lupus erythematosus retaining lupus anticoagulant (LAC), we established 6 Epstein-Barr virus-transformed human B cell clones secreting antibodies that affect the coagulation assay. Two and 4 of the clones secreted IgM and IgG antibodies, respectively. Although all 6 antibodies displayed anticardiolipin activity in ELISA, the increased binding activity in the presence of β_2-glycoprotein I was limited only to the IgG antibodies. Five antibodies (two IgM and three IgG) had LAC activity which prolonged the activated partial thromboplastin time (APTT), whereas one IgG antibody shortened the APTT. Two of the IgG producing clones had an identical Ig heavy chain gene rearrangement despite their opposite effects on the coagulation assay. These results demonstrated the heterogeneity of LACs and diversity among their physiological functions.

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Lupus anticoagulant (LAC), which was first described as a natural anticoagulant factor in patients with systemic lupus erythematosus (SLE), is an autoantibody directed against phospholipids including cardiolipin. It shows anticoagulant activities in phospholipid dependent coagulation assays such as the activated partial thromboplastin time (APTT). However, patients with LAC usually do not have bleeding diathesis but rather paradoxically exhibit a tendency toward thrombosis (1). Many investigators have indicated a close relationship between LAC and adverse pregnancy outcomes due to thromboembolism in the placenta. These include spontaneous abortion, intrauterine growth retardation and recurrent still birth (1, 2). Since pathogenic autoantibodies in autoimmune diseases are considered to be IgG, early studies have mainly focused on the involvement of IgG class LAC or anticardiolipin antibody (ACA) in gestational impairment (2-4). Harris et al. (3) have reported that the high level of IgG ACA is predictive of fetal loss. Branch et al. (4) described that pregnant mice injected with IgG ACA aborted within 48 hours and that the deciduae of the aborted mice showed considerable IgG and fibrin deposition.

Although understanding of the clinical features of LAC has increased, the diversity of LACs and the difference between IgM and IgG LACs in pathogenesis have remained controversial. For example, Gleicher et al. (5) have reported the association of IgM LACs with habitual abortion. To address these issues, the physiological nature of IgM and IgG LACs from individual patients should be compared at the monoclonal level. Recently, we established 6 Epstein-Barr virus (EBV) -transformed B cell clones secreting antibodies that affect the APTT assay. Using the established clones, we examined the effects on the coagulation assay and interactions between the antibodies.

Materials and Methods

The patient is a 34-yr-old woman with SLE who satisfied the revised criteria of the American Rheumatism Association (6). She has experienced Libman-Sack endocarditis, glomerulonephritis, seizure attacks and six consecutive fetal losses but no history of bleeding diathesis. At sampling, she was not pregnant and was in the relapsing phase under no drug administration. Her laboratory examination revealed biological false positive serologic tests for syphilis, the presence of ACA in a β_2-GPI dependent manner and remarkable prolongation of APTT, which was not corrected by adding an equal volume of normal plasma. Details and conditions of the EBV-transformation, cloning and Southern blot procedures were essentially the same as described elsewhere (7).

LAC positive clones were screened using culture supernatants, by means of the APTT assay described by Rauch et al. (8) All samples were tested in duplicate, and the mean values were adopted. Platelin® (Organon Teknika Corp. , Durham, NC, USA) diluted 20-fold was used as the phospholipid source. The culture supernatants, the concentrations of which varied from 4 to 7 μg/ml, were also assayed by human IgM- or IgG- specific ELISA for heavy chain isotype determination. The supernatants of clones C2 and C18 were used as IgM and IgG controls, respectively. To determine the interactions between the monoclonal antibodies, the samples assayed by APTT consisted of pairs of supernatants from different clones, at equal volumes. Antibodies from the selected clones were tested for cardiolipin binding activity by ACA ELISA in the presence (10 μg/ml) or absence of β_2-GPI; our ACA ELISA followed the procedure of McNeil et al. (9) Each of the culture supernatants of the selected clones was added to 6 wells (50 μl/well) in one ELISA plate (coated with 50 μg/ml cardiolipin). Alkaline phosphatase

(ALP) -conjugated goat antihuman IgM or IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) diluted 500-fold in 0.05% Tween 20/phosphate buffered saline (PBS-T) was used as the second antibody depending on the Ig class of antibodies tested. Every wash was performed three times with PBS-T. P-nitrophenyl phosphate in bicarbonate buffer was used as a substrate for ALP. Optical density (O. D.) values read at 405 nm were statistically analyzed (t-analysis). APTT in seconds and the O. D. of all samples were compared with those of cell culture medium, RPMI 1640 + 10% fetal calf serum (RPMI/FCS).

Results

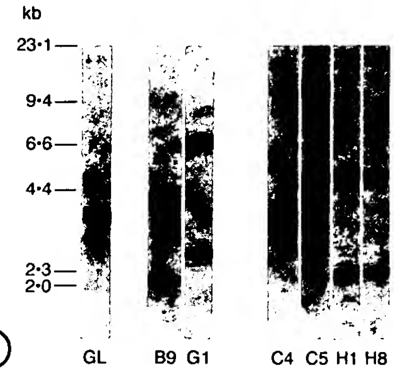
We finally analyzed 6 EBV-transformed human B cell clones established from one patient with SLE. Two clones (B9 and G1) secreted IgM, while the other four (C4, C5, H1 and H8) produced IgG (Table 1). To confirm the monoclonality and to compare the Ig heavy (H) -chain gene rearrangements of the clones, we performed Southern hybridization using a human J_H probe. As shown in Fig. 1, all of the Bgl II digested genomic DNAs from the 6 clones generated only two rearranged J_H-containing DNA fragments of various sizes, which demonstrated the monoclonality of each clone. An identical profile of IgH-chain gene rearrangement was found in H1 and H8 DNAs, although the supernatants from these two clones exert opposite effects ,namely, H1 prolonged APTT whereas H8 shortened it (described below). The possibility of comigration of different J_H-containing DNA fragments was examined by means of digestion with various restriction endonucleases. The same results were repeatedly obtained from Hind III (Fig. 2A), Eco RI (Fig. 2B) or Bam HI (data not shown) digests, suggesting that these two clones are clonally related.

The supernatants of the 6 clones were tested for the activities of anticoagulant and cardiolipin binding by APTT assay and by ELISA, respectively. The supernatants from 5 clones (B9, G1, C4, C5 and H1) had LAC activity that prolonged APTT and which worked synergistically (Tables 1 and 2). The supernatant from clone H8 shortened the APTT and antagonized the LAC activities of the other 5 supernatants (Tables 1 and 2). The ELISA showed that all of the 6 supernatants carried cardiolipin binding activity (Table 1). The APTTs and O. D. s of supernatants from C2 or C18 did not show significant changes in comparison with those of RPMI/FCS. ACAs related to thromboembolism are reportedly directed against

Table 1 : ACA ELISA of the supernatants from 6 lymphocyte clones

clone	isotype	O.D. in ACA ELISA*
B9	IgM	0.48 ± 0.08
G1	IgM	0.79 ± 0.04
C4	IgG	0.17 ± 0.02
C5	IgG	0.35 ± 0.02
H1	IgG	0.71 ± 0.17
H8	IgG	0.67 ± 0.15

*Values are means $\pm$SD of 6 wells, in which O.D. with RPMI/FCS was set to zero.



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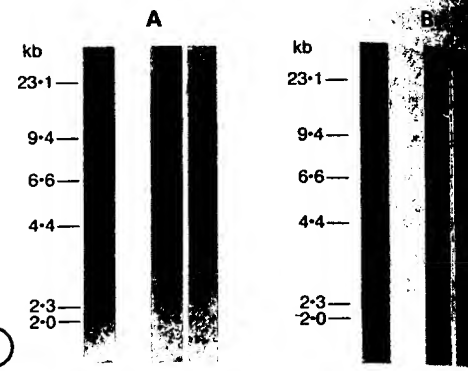

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Fig. 1.
Genomic Southern hybridization of Bgl II -digested DNAs from 6 B lymphocyte clones.
Molecular size is expressed in kilobases (kb). GL is a germ line control.

Fig. 2.
A comparison of the Southern hybridization profiles between clones H1 and H8.
The DNAs were digested with Hind III (A) or Eco RI (B).

an antigen composed of a complex of negatively charged phospholipids and cardiolipin cofactor (β_2-GPI) (9). In ELISA, β_2-GPI increased the cardiolipin binding activities of IgG antibodies from clones C4, C5, H1 and H8, but decreased those of IgM antibodies from clones B9 and G1 (Fig. 3).

Discussion

So far, limited information is available concerning the characteristics of LAC antibodies, since most LACs have been analyzed using sera, IgM or IgG fractions from patients. B cell clones producing antiphospholipid antibodies have been established from patients with SLE (8, 10). However, most of them secrete IgM (8), and only one known IgG class antibody does not have LAC activity (10). Therefore, the present report is the first description of the

Table 2 : Interactions between monoclonal antibodies in the APTT

	B9	G1	C4	C5	H1	H8
B9	76.3	88.9	89.8	87.2	80.6	70.5
G1		79.8	89.0	87.7	81.8	71.9
C4			82.7	90.2	83.9	73.6
C5				78.7	84.5	74.6
H1					77.8	72.1
H8						55.1

Numbers refer to the APTT in seconds (mean of duplicate).
The APTT with RPMI/FCS was 63.9 seconds.
Supernatants of different clones were mixed in pairs (at equal volumes) and the APTT was assayed.

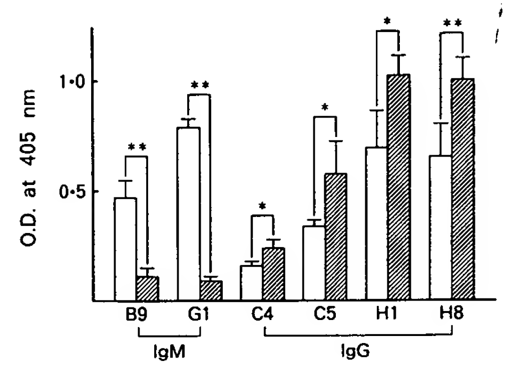

Fig. 3.
The binding activities of monoclonal antibodies from 6 clones to cardiolipin in ELISA. The dependence or independence upon ß$_2$-glycoprotein I of the cardiolipin binding of the 6 human monoclonal antibodies was tested using culture supernatants. ACA ELISA was performed in the presence (hatchedcolumn) or absence (unfilled column) of ß$_2$-glycoprotein I. *P < 0·005, **P < 0·001.

heterogeneity of LACs and of a difference in the function among antibodies, in particular, between IgM and IgG LACs in an individual.

We established 6 EBV-transformed human B cell clones that produce monoclonal IgM or IgG antibodies that affect phospholipid dependent coagulation assay. Among them, 5 antibodies (B9, G1, C4, C5 and H1) prolonged the APTT and worked synergistically regardless of their isotypes (Tables 1 and 2). The distinct Southern blot profiles of IgH-chain gene rearrangement among the B cell clones gave direct proof of the heterogeneity of LACs in an individual. The synergistic effect of heterogeneous LACs may be related to the variations in thromboembolism.

One antibody, H8, shortened the APTT and inhibited the LAC activity of the other 5 antibodies (Tables 1 and 2). This is the first identification of such an antibody in LAC positive patients. Although its physiological function remains unknown, similar findings in Graves' disease, in which thyroid-stimulating antibody (TSAb) and thyroid-stimulation-blocking antibody (TSBAb) coexist in patient sera (11), have been reported.

In addition to their physiological activities in the coagulation assay, all 6 antibodies displayed cardiolipin binding activity (Table 1). The increment in the binding of IgG antibodies to cardiolipin in the presence of ß$_2$-GPI (Fig. 3) is consistent with previous observations, supporting the involvement (12-14) of ß$_2$-GPI in the antigen recognition of pathogenic IgG ACA. The binding activity of two IgM antibodies to cardiolipin was inhibited by ß$_2$-GPI. Previous studies have reported that the binding activity to cardiolipin of serum autoantibodies from patients with infectious diseases (12), syphilis (13) or hemophiliacs (14) was inhibited by ß$_2$-GPI. However, there are no reports about the inhibitory effect of ß$_2$-GPI upon the binding of human monoclonal IgM autoantibody with LAC activity to cardiolipin. Our results imply that the features of binding activity to cardiolipin by LACs are divided into two types. One is

dependent upon β_2-GPI, as shown in our IgG LACs, and the other is not dependent upon β_2-GPI, as shown in our IgM LACs. This typing probably results from a difference in the recognized epitopes between IgM and IgG LACs in this patient, which should be clarified.

Of interest is that two IgG producing clones, H1 and H8, displayed an identical profile of IgH-chain gene rearrangement, indicating that they are clonally related. Although both clones possess cardiolipin binding activity in a β_2-GPI dependent manner, they had opposite effects on the coagulation assay. These results indicated the contribution of somatic mutations to the alteration of physiological phenotypes of LAC antibodies.

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Review

Int Arch Allergy Immunol 1995;108:103–112

Interleukin-12

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Key Words
Cytokines
Th1/Th2
Autoimmunity
Infectious diseases
Adjuvants
Receptor antagonists

Abstract
Interleukin (IL)-12 was originally identified as a factor produced by human Epstein-Barr virus-transformed B cell lines. It was detected by one group as cytoxic lymphocyte maturation factor, a cytokine that synergized with IL-2 in the induction of lymphokine-activated killer cells and cytotoxic T lymphocytes. A second group characterized it as a natural killer (NK) cell stimulatory factor, due to the enhancement of cytotoxicity and IFN-γ synthesis by NK cells. Human IL-12 was purified to homogeneity and cloned by both groups. We had identified a murine factor, provisionally termed T cell-stimulating factor (TSF), which was involved in the proliferation, synthesis of IFN-γ and cell adhesion of CD4+ Th1 cells. TSF was produced in the antigen-specific interaction between Th1 cells and macrophages as antigen-presenting cells, partially purified from supernatants of such cultures, and shown to be identical to IL-12. Monocytes/macrophages appear to be the major source of IL-12. It is rapidly produced by phagocytic cells after stimulation with several bacteria/bacterial products and other microorganisms. In the light of its effects on NK cells as well as CD4+ and CD8+ T cells, IL-12 can be regarded as a cytokine that connects the innate immune system with the acquired immunity. IL-12 has a broad range of activities already reviewed in three papers. These include the regulation of cytokine synthesis and proliferation of T and NK cells, the promotion of Th1 cell development, the differentiation of CD8+ T cells and effects on hematopoiesis. When applied in vivo, IL-12 was shown to enhance the resistance to bacterial and parasitic infections, to promote antitumor immunity, and to influence antiviral responses including HIV in vivo or in vitro. This review will briefly summarize these effects, but mainly focus on recent results concerning the regulation of the production and the activity of IL-12, its role in the differentiation of Th cells and the implications for delayed- and immediate-type hypersensitivity reactions, its importance for organ-specific autoimmune diseases, and the possible role of the IL-12p40 homodimer as a specific inhibitor of the IL-12 heterodimer.

Structure of IL-12 and Its Receptor, Signal Transduction and Role of IL-12(p40)$_2$

The heterodimeric structure of IL-12 is unique among cytokines [1–3]. Two N-glycosylated polypeptide chains of approximately 40 kD (p40) and 35 kD (p35) linked by a single disulfide bond form the active molecule. Mature human p40 is composed of 306 amino acids with a calculated molecular weight (M_r) of 34,699. It contains 10 cysteines and 4 possible N-linked glycosylation sites. The mature human p35 is composed of 197 amino acids with a calculated M_r of 22,513. It contains 7 cysteines and 3 possible N-linked gly-

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cosylation sites [4, 5]. The genes encoding both chains of murine IL-12 were cloned by cross-hybridization using human cDNA clones [6]. The murine p40 chain has 70%, the murine p35 chain 60% identity to the human genes, respectively. Coexpression of both chains of IL-12 within one cell is required to generate bioactive IL-12 heterodimer [4–6]. Whereas murine IL-12 is active on mouse and human cells, human IL-12 is only active on human cells. This species specificity appears to be determined by the p35 chain, because a hybrid heterodimer consisting of murine p35 and human p40 retained activity on murine and human cells. In contrast, a hybrid IL-12 molecule comprised of human p35 and mouse p40 was active on human but not on mouse cells [6]. Certain parts of the IL-12p35 sequence are related to those of IL-6 and granulocyte colony-stimulating factor (G-CSF), suggesting that p35 is evolutionary derived from a primordial cytokine [7]. The primary amino acid sequence of p35 predicts a structure containing 4 antiparallel α-helices. By replacing murine p35 sequences with human p35 sequences, Zhou et al. [8] identified five sites clustered on one side of the p35 molecule which contribute to the species specificity of IL-12. Such a mutated molecule was still active on human cells. However, the affinity for the mouse IL-12 receptor was about 30-fold lower and the bioactivity was reduced about 3,000-fold, demonstrating that p35 significantly contributes to receptor binding and largely determines bioactivity [8]. In contrast, IL-12p40 appears to be mainly involved in receptor binding without inducing a biological signal. This is supported by the finding that supernatants of COS cells transfected with the IL-12p40 cDNA specifically antagonized in a dose-dependent manner various effects of the IL-12 heterodimer on murine Th1 clones [9]. Gillessen et al. [10] have shown that a homodimer of the murine p40 chain [IL-12(p40)$_2$] binds to the high affinity IL-12 receptor with an affinity similar to that of the IL-12 heterodimer, whereas the p40 monomer is about 25- to 50-fold less active than the homodimer in causing specific, dose-dependent inhibition of IL-12 in bioassays as well as in competitive binding studies of [^{125}I]-labeled mouse IL-12 to T cell blasts. Human p40 also exists in a monomeric and dimeric form, the latter being about 20-fold more effective in inhibiting the IL-12 heterodimer. However, in contrast to mouse IL-12(p40)$_2$, the affinity of human IL-12(p40)$_2$ for the high-affinity IL-12 receptor is 5- to 10-fold lower than that of human IL-12. Likewise, human IL-12(p40)$_2$ is approximately 10-fold less potent than mouse IL-12(p40)$_2$ in the inhibition of biological effects of the IL-12 heterodimer [11]. The human p40 sequence is related to the extracellular domain of some members of the hematopoietic cytokine receptor superfamily, in particular to the IL-6 receptor (IL-6R) [12] and the ciliary neurotrophic factor receptor [6]. The observed relatedness between p35 and G-CSF as well as between p40 and the IL-6R led to the suggestion that IL-12 might be derived from a complex of a cytokine with its soluble receptor, and that the IL-12 receptor might have some homology to the gp130 molecule [7], which is part of the high-affinity IL-6R and responsible for signal transduction [13]. Indeed, the recent cloning of a human IL-12 receptor chain confirmed this hypothesis. The cDNA encodes for a 662-amino acid type I transmembrane protein which has significant homology to gp130 [14]. The extracellular domain comprises 516 amino acids and is separated by a transmembrane part from the 91-amino acid cytoplasmic domain. However, this subunit, probably as dimer or even oligomer, binds IL-12 and IL-12(p40)$_2$ with only low affinity (2–6 nmol/l). Thus, further IL-12R subunits probably exist which are required for the intermediate (50–200 pmol/l) and high-affinity (5 to 20 pmol/l) binding of IL-12 to phytohemagglutinin-activated peripheral blood mononuclear cells (PBMC) [14]. The cloned IL-12R subunit does not contain motifs known to be involved in the initiation of signal transduction. Tyrosine kinases appear to be activated following stimulation of T and NK cells with IL-12 [15–17]. These include JAK2 and TYK2 kinase [17] as well as a 44-kD mitogen-activated kinase [16].

Regulation of IL-12 Synthesis and Activity as well as Expression of the Subunits

Human IL-12 was originally identified and purified from the supernatants of Epstein-Barr virus (EBV)-transformed B cell lines stimulated with phorbol diesters [18–20]. Most but not all lines from a large panel of EBV-transformed B cells produced IL-12, whereas Burkitt-lymphoma-derived lines, even when expressing EBV, did not [20]. However, normal B lymphocytes do not appear to be a major source of IL-12 in vivo, since injection of lipopolysaccharide (LPS) yields similar levels of IL-12 in normal and in B cell-deficient SCID mice [21]. Furthermore, IL-12 is not produced when Th1 cells are activated with B cells as antigen-presenting cells (APC) [unpubl. observation, indirect evidence in 22, 23]. The major producer cells of IL-12 were found to be monocytes/macrophages. Upon stimulation with LPS, gram-positive and gram-negative bacteria [20, 24–26], and intracellular parasites [27, 28] monocytes/macrophages efficiently produce IL-12. Very recently, a *Leishmania major*-derived recombinant protein was described which induced the synthesis of IL-12 by human PBMC as well as by purified monocytes [29]. Besides various known and unknown

products from different microorganisms as triggers of IL-12 synthesis, mouse IL-12 was originally identified as a factor produced during the antigen-specific interaction of Th1 cells and spleen cells or bone marrow-derived macrophages as APC [30]. IL-12 synthesis in such cultures did not necessarily depend on the antigen-specific interactions because cocultivation of activated [with an anti-CD3 monoclonal antibodies (mAb) or with antigen presented by major histocompatibility complex (MHC) class II-transfected fibroblasts] Th1 cells and allogeneic, third party macrophages did also result in the production of IL-12. Direct contact between the activated T cells and the macrophages was required, suggesting that membrane molecules are involved. Shu et al. [31] very recently reported similar findings. In addition, they showed that interfering with the CD40-CD40 ligand (gp 39) interaction in cocultures of monocytes and activated Th cells abrogated the synthesis of IL-12. Furthermore, ligation of CD40 molecules on the surface of monocytes by cross-linked anti-CD40 mAb resulted in IL-12 production. These results strongly support the conclusion that the interaction of the CD40 ligand (gp 39) expressed on activated T cells with CD40 on the surface of monocytes/macrophages results in the synthesis of IL-12. It has been reported that bacterial superantigens such as staphylococcal enterotoxin B and toxic shock syndrome toxin-1 induce IL-12 production by human PBMC [32]. However, since whole PBMC but not purified monocytes were activated with these superantigens it is unclear whether this is a direct effect on monocytes or indirectly mediated by activated T cells. Besides monocytes/macrophages other cell types have also been reported to produce IL-12 or to express mRNA for both subunits of IL-12. These include dendritic cells [33, 34], neutrophils activated with LPS in the presence of interferon-γ (IFN-γ) [35], normal and malignant keratinocytes [36, 37], murine mast cells cultured in mast cell growth factor [38] and thymic stromal cells [39]. The most potent inhibitor of IL-12 synthesis by monocytes/macrophages appears to be IL-10 [23, 24, 26, 27, 35, 40–43], which acts at the protein as well as at the mRNA level. The cytokines IL-4, IL-13 and transforming growth factor-β (TGF-β) can also suppress the production of IL-12 when added simultaneously with bacterial inducers. However, pretreatment of monocytes with IL-4 or IL-13 followed by activation with *Staphylococcus aureus* Cowan (SAC) or LPS enhances IL-12 production [44]. Similar to, but independent of IL-10, addition of prostaglandin E_2 to cultures of LPS-stimulated human PBMC strongly downregulated IL-12 production [45]. We have made similar observations [unpubl. data] using the phosphodiesterase inhibitor pentoxifylline and mouse macrophages stimulated with LPS or heat-killed Listeria. Thus, elevations of the intracellular levels of cyclic AMP can also inhibit the synthesis of IL-12 by monocytes/macrophages.

The ability of recombinant IL-12$(p40)_2$ to inhibit the bioactivity of heterodimeric IL-12 might constitute a second level of regulation. Experiments with recombinant IL-12$(p40)_2$ indicate that an about 10- or 100-fold excess of IL-12$(p40)_2$ over IL-12 is required to achieve a 50 or 90% inhibition of the effects of IL-12 on Th1 cells, respectively [unpubl. observations]. Stimulation of EBV-transformed B cell lines with phorbol diesters [18–20] and of human PBMC [20], human peripheral blood neutrophils [35], or mouse macrophages [unpubl. observations] with bacterial products resulted in the production of a large (10- to 100-fold) excess of IL-12p40 over IL-12 heterodimer. Furthermore, normal mouse serum as well as sera from mice injected with LPS [46] and pleural fluid from humans with tuberculous pleuritis [47] have been shown to contain p40 in excess over the IL-12 heterodimer. In situ hybridization studies on spleen sections from either unstimulated or LPS-injected mice revealed that most of the mRNA for p35 and p40 were localized in different, mutually exclusive areas of the spleen [48]. This suggests that p40 might be produced in vivo, either by cells expressing only p40 and no p35 or possibly by cells which first synthesize IL-12 but continue to upregulate p40 expression leading to an overproduction of IL-12p40. However, whether this is mainly IL-12$(p40)_2$ or monomeric p40, and whether the overproduction of p40 is of physiological relevance has yet to be demonstrated.

A third level of regulation of the bioactivity of IL-12 is probably exerted by TGF-β. This potent anti-inflammatory cytokine inhibited the IL-12-induced cytokine production by naive human CD4+ T cells [49], the IL-12-promoted Th1 cell development of naive murine CD4+ T cells [50], and the IL-12-triggered synthesis of IFN-γ by murine natural killer (NK) cells as well as the T cell-independent resistance of mice to *Toxoplasma gondii* infection [51].

While studying the mRNA expression of both subunits of IL-12, it was observed that p35 transcripts were ubiquitously expressed in almost all hematopoietic and nonhematopoietic cell lines investigated [1]. Furthermore, only p35 but no p40 message was detected in mouse brain and lung [6]. In addition, the B cell regions in spleens from normal or LPS-injected mice showed a broad staining for p35 but almost no p40 signal when investigated by in situ hybridization [48]. Since to date secretion of the p35 protein by normal cells or cell lines has not been demonstrated, the significance of the expression of p35 transcripts in the absence of p40 remains to be determined.

Effects of IL-12 on Proliferation, Cytokine Production and Cytotoxicity by T and NK Cells

Resting T and NK cells show little if any proliferation in response to IL-12. Activation of PBMC with lectins, phorbol diesters, anti-CD3 mAb, allogeneic cells or IL-2 induces responsiveness to IL-12. This can in part be explained by an upregulation of receptors for IL-12 [52]. Growth-promoting effects of IL-12 were described for CD4+ and CD8+ T cells [22, 23, 53–57], γ/δ T cells [55], and NK cells [53, 58]. These can be direct, as shown for CD8+ T cell blasts shortly after activation (day 2–6), or due to the at least additive growth-promoting activity of IL-2 and IL-12 on more resting T cells [22, 23, 53–55]. When activated with antibodies against CD28, the proliferation of human lymphocytes was synergistically enhanced by IL-12 [43]. Likewise, the proliferation of murine Th1 clones activated with splenic APC was dependent on IL-12 and B7 [42]. Anergized murine T cells did only marginally proliferate to IL-12 due to a lack in IL-12 responsiveness [59]. In contrast to these growth-enhancing effects, IL-12 can also suppress the proliferation of resting γ/δ T cells and NK cells [55, 56, 60] induced by high doses of IL-2.

One of the most important effects of IL-12 is the strong enhancement of IFN-γ synthesis by T and NK cells. This effect is observed even with resting cells expressing only a few high-affinity IL-12 receptors. In vivo, intraperitoneal injection of mice with IL-12 also results in strong IFN-γ production [61]. IL-12 enhances the synthesis of IFN-γ by human [18, 49, 62] and mouse [9, 63] T cells induced with phorbol diesters of T cell receptor/CD3. A particularly strong synergistic effect on the production of IFN-γ by T and NK cells was observed when IL-2 and IL-12 were combined [9, 18, 49, 62]. IL-12 primarily upregulated the transcription of the IFN-γ gene, whereas the effect of IL-2 was mainly posttranscriptionally [64]. In addition to IL-2, the cytokines tumor necrosis factor-α (TNF-α) and IL-1β [40, 49] as well as the interaction of CD28 with the B7 ligands enhance the IL-12-triggered synthesis of IFN-γ [42, 43]. Besides IFN-γ synthesis, activation of human T cells with IL-12 and anti-CD28 antibodies resulted in the synthesis of granulocyte-macrophage colony-stimulating factor and TNF-α [43], indicating that IL-12 can affect the production of several cytokines.

IL-12 also exerts profound effects on the cytotoxicity of T and NK cells. Activation of NK cells (generation of lymphokine-activated killer cells) with IL-12 augmented the cytotoxic activity against NK cell-sensitive and cell-resistant target cells in vitro [15, 60, 65–67] and in vivo [61]. TNF-α appears to be an important cofactor for the IL-12-induced development of LAK cell activity [65, 66]. The maturation as well as the the cytotoxic activity of CD8+ T cells is enhanced by IL-12 [15, 56, 57, 68]. The expression of the cytotoxicity-associated proteins granzyme A and B as well as perforin is upregulated by IL-12 in T and NK cells [57, 68, 69].

Taken together, these results clearly show that IL-12 has a profound effect on proliferation, cytokine synthesis, and cytotoxicity of T and NK cells [see also 3], the main target cells of IL-12. Some recent evidence suggests that IL-12 may also act (directly) on B cells and APC. The secretion of IgG by purified human B cells activated with SAC and IL-2 was increased by IL-12 [70], and preincubation of APC (splenic adherent cells) with IL-12 inhibited their capacity to induce the antigen-specific synthesis of IL-4 by in vivo-primed memory T cells [71].

The Influence of IL-12 on Hematopoiesis

IL-12 has a direct effect on early hematopoietic progenitor cells. In combination with other hematopoietic growth factors such as steel factor, IL-3 or GM-CSF it promotes the growth/survival of hematopoietic progenitor cells in vitro [72–74] and it induces extramedullary hematopoietic foci in the spleen and liver of IL-12-treated mice [61]. However, in the presence of a few NK cells it inhibits colony formation in vitro due to the secretion of TNF-α and IFN-γ [74], and prolonged treatment with high doses of IL-12 in vivo results in severe anemia and neutropenia [61].

IL-12 in Infectious Diseases

Similar to TNF [75, 76], IL-12 has a central role in the immune response to several microorganisms. IL-12 is part of the innate resistance mechanisms of the immune system, as shown by the ability of anti-IL-12 reagents to interfere with the response of SCID mice to infection with *Listeria monocytogenes* [77] or *T. gondii* [78]. It is also part of the acquired immune response to different microorganisms, mediated largely by CD4 T cells. Antibodies to IL-12 inhibited the protective immune response to *L. major* [79], *Listeria m.* [77], *T. gondii* [78], *Yersinia enterocolitica* [80], and *Candida albicans* [81] in the respective mouse strains. Furthermore, administration of IL-12 to susceptible strains of mice, which usually succumb to infection, induced a protective immune response. This occurred in BALB/c mice infected with *L. major* [79, 82, 83] or *Y. enterocolitica* [80] and in C57/BL6 mice immunized with *Schistosoma mansoni* eggs [84, 85]. IL-12 treatment also prolonged the survival of

immunodeficient mice infected with *T. gondii* [28, 86]. Even more important was the potent adjuvant effect of IL-12 in the prophylactic immunization with microbial proteins. Vaccination of BALB/c mice with leishmanial antigen and IL-12 or of C57/BL6 mice with *S. mansoni* eggs and IL-12 resulted in protection against a subsequent challenge with *L. major* [87] or *S. mansoni* eggs [84]. In infectious diseases of humans, IL-12 was detected in the pleural fluid of patients with tuberculous pleuritis [47] and it was expressed 10-fold greater in lesions of patients with tuberculoid leprosy than with lepromatous leprosy [88]. Neutralization of IL-12 partially inhibited the mycobacteria-specific proliferation of pleural fluid cells [47], and exogenous IL-12 stimulated proliferation by CD4+ T cells from patients with tuberculoid leprosy but not by CD8+ T suppressor cells from patients with lepromatous leprosy [88; for a more detailed review, see 3].

IL-12 in Murine Shock Models

Whereas TNF and IL-12 are necessary in the defense against microorganisms, an overproduction of both cytokines plays a central role in shock pathogenesis. In LPS-triggered endotoxic shock [21, 46] as well as in the generalized Shwartzman reaction [89] neutralization of IL-12 prevented the lethal outcome of shock, primarily by inhibiting the synthesis of IFN-γ.

IL-12 in Viral Infections Including HIV

Administration of IL-12 to mice infected with the lymphocyte choriomeningitis virus (LCMV) has opposite, dose-dependent effects [90, 91]. Daily doses of 10 or 1 ng IL-12 reduced the LCMV titer. In contrast, higher doses (0.1/1 μg/day) suppressed the proliferation of LCMV-specific CD8+ T cells and led to an increase of the LCMV titer. The detrimental effects of high doses of IL-12 in LCMV infection were largely prevented by neutralization of TNF-α [91]. However, treatment with IL-12 inhibited the development of a retrovirus-induced syndrome termed murine acquired immunodeficiency syndrome (MAIDS). IL-12 treatment was effective in MAIDS when started at the time or even several weeks after infection and required endogenous IFN-γ [92]. Similarly, in human HIV infection/AIDS treatment with IL-12 might be beneficial. PBMC from HIV-infected patients produced less IL-12 in response to bacterial stimuli than PBMC from healthy donors [93]. Addition of IL-12 to PBMC from AIDS patients enhanced the synthesis of IFN-γ and the proliferation of T cells to recall antigens and HIV peptides [94] as well as the cytotoxic activity of NK cells [67]. Thus, IL-12 might be able to enhance an anti-HIV immune response as well the resistance to opportunistic infections frequently observed in AIDS patients.

Tumor Models and IL-12

Systemic treatment with relatively high doses of IL-12 [95–97], or stable transduction of tumor cells with the genes for both subunits of IL-12 [98] can suppress the growth and metastasis of certain tumors in several in vivo models. In some murine tumor models, therapeutic activity of IL-12 against already established tumors was observed [95–97]. Low doses of IL-12 in combination with a tumor-specific peptide in adjuvant elicited a protective antitumor response [97]. However, the molecular and cellular events mediating the IL-12-induced antitumor response are complex. Whereas in some models the protective effect appears to be largely mediated by CD8+ T cells [95–97], NK cells were mainly responsible in another model [98]. Neutralization of IFN-γ can reverse the antitumor activity of IL-12 [96]. In one model, depletion of CD4+ T cells strongly enhanced the NK-/CD8+ T cell-mediated antitumor response [98]. In contrast CD4+ T cells were involved in the protective effect of tumor peptide + IL-12 treatment [97].

IL-12 and Organ-Specific Autoimmune Diseases

There is increasing evidence that organ-specific autoimmune diseases such as diabetes in nonobese diabetic mice or experimental allergic encephalomyelitis (EAE) are mediated by Th1-type cells and that Th2-type cells might be protective [99]. This is further supported by the observations that administration of IL-12 induced a more prolonged and severe form of EAE in an adoptive transfer model with primed, autoreactive lymph node cells [100], accelerated the onset of diabetes in female nonobese diabetic mice [101], and induced a severe, destructive arthritis in DBA/1 mice immunized with type II collagen in incomplete Freund's adjuvant [102]. Furthermore, anti-IL-12 antibodies reduced the incidence and severity of EAE in the adoptive transfer model [100] and prevented the development of diabetes in a transgenic disease model following transfer of specific T cells from diabetic mice [103]. This shows that anti-IL-12 treatment is still effective when already differentiated T(h1) cells are transferred and may reflect the importance of the ability of Th1 cells to induce the production

of IL-12 in monocytes/macrophages as well as the potent costimulatory activities of IL-12 on Th1 cells [22, 23, 31, 42, 43, 63]. The importance of IL-12 at later stages of an autoimmune response is clearly documented in a mouse model of inflammatory colitis. Established colitis was successfully treated with an anti-IL-12 mAb, as monitored by an increase in body weight and abrogation of IFN-γ synthesis by lamina propria CD4+ T cells [104].

Effects of IL-12 on the Differentiation of Th Cells, the Humoral Immune Response, and Implications for Immediate-Type and Delayed-Type Hypersensitivity Reactions

One of the most striking, and probably clinically relevant, effects of IL-12 is its ability to promote the development of Th1-type immune responses. This was observed in vitro, using mouse [24, 50, 105] or human T cells [49, 106, 107], as well as in several mouse models of infection [77–81, 84, 87, 108] or immunization [109, 110]. Therefore, IL-12 was termed an 'initiation cytokine for cell-mediated immunity' [111]. The ability of IL-12 to inhibit many Th2-type responses in vivo [79, 80, 82, 84, 108] and the strong suppression of IgE production in vitro [112] as well as of antibody production in vivo [109, 113] supports this view. However, the influence of IL-12 on the differentiation of Th cells as well as on humoral immune responses is much more complex. In BALB/c mice infected with *L. major*, early treatment with IL-12 prevents the development of the Th2 response [79, 82], which would occur in the absence of exogenous IL-12, by direct effects independent of IFN-γ [83]. Generation of T cell lines from bulk cultures of PBMC (plus allergen) from atopic humans in the presence of IL-12 strongly suppressed their ability to produce IL-4. This inhibitory activity of IL-12 was also observed if an anti-IFN-γ mAb was added [106]. Likewise, addition of IL-12 to cultures of murine CD4+ T cells, isolated from the draining lymph nodes of immunized mice and reactivated with antigen+APC in vitro, profoundly reduced the synthesis of IL-4. IL-12 appears to act via the APC and, again, independently of IFN-γ [71]. In contrast, the downregulation of IL-4 synthesis in mice immunized with *S. mansoni* eggs and treated with IL-12 was no longer observed when IFN-γ was neutralized [84, 85]. The strong inhibition of IgE synthesis and Th2-type cytokine production in IL-12-treated BALB/c mice infected with *Nippostrongylus brasiliensis* also required the IL-12-induced upregulation of IFN-γ synthesis [108]. Thus, IL-12 by direct and indirect (IFN-γ-mediated) effects can inhibit Th2/IgE responses. However, when the IL-12 treatment of *L. major*-infected BALB/c mice was delayed for 2 weeks, IL-12 no longer suppressed but rather enhanced the Th2/IL-4 as well as the Th1/IFN-γ response [79, 83]. Delayed treatment with IL-12 also fails to inhibit the Th2/IgE response of mice immunized with nematode parasites [108] or injected with goat antimouse IgD [113]. Even more surprising, in at least two animal models, CBA/J mice immunized with protein antigens adsorbed to aluminum hydroxide [114] and in nonhealer strains of mice infected with *C. albicans* [81], IL-12 treatment started with the first immunization/infection failed to (permanently) suppress the IgE/Th2 response, although it induced T cells producing IFN-γ. These in vivo results probably reflect in vitro data by several groups demonstrating that IL-12 failed to inhibit [24, 105, 107, 115, 116] or even supported [115, 116], the generation of IL-4-producing T cells when both IL-12 and IL-4 were added during the initial priming of the T cells. However, this implies that in both of these murine in vivo models at least some of the antigen-/pathogen-specific T cells can produce IL-4 upon primary activation. This would be similar to the results obtained with human neonatal (naive) CD4+ T cells in vitro. Addition of IL-12 (without exogenous IL-4) to anti-TCR-activated T cells promoted the development of T cells producing high levels of IL-4 in addition to IFN-γ [117].

Besides its role in the development and activation of Th cells, IL-12 also has a profound effect on humoral immune responses. Whereas the initial reports described mainly inhibitory effects of IL-12 on the synthesis of IgE by polyclonally activated human B cells in vitro [112] as well as polyclonal [113] or antigen-specific [109] IgE, IgG1 and IgG2b synthesis in vivo, three recent reports decribed a potent stimulatory influence of IL-12 on the synthesis of antibodies. In CBA/J mice immunized with protein antigens adsorbed to alum [110], administration of IL-12 induced 10- to 1,000-fold increases in antigen-specific IgG2a, IgG2b and IgG3 production. IgG1 synthesis was not suppressed but rather slightly enhanced. Even the synthesis of IgE was slightly enhanced when low doses of IL-12 were injected [114]. Furthermore, the titers of collagen-specific antibodies were much higher in the sera of DBA/1 mice immunized with type II collagen in incomplete Freund's adjuvant and simultaneously treated with IL-12, as compared to the serum titers of mice which did not receive IL-12 [102]. Moreover, in combination with IL-2, IL-12 strongly enhanced Ig synthesis by highly purified, polyclonally activated human B cells [70]. The influence of IL-12 on humoral immune responses might be due to direct effects on B cells [70] or to the generation of potent T helper cells such as the CD30+ subset of human T cells [118].

Taken together, the effects of IL-12 on the development of Th2 cells as well as on antibody synthesis including IgE are complex. Further studies are required before the question of whether IL-12 has some potential in the prevention or treatment of immediate-type allergic disorders/atopy can be answered.

In contact sensitivity, a classical Th1-type response in the skin, treatment with an anti-IL-12 antiserum prior to epicutaneous allergen painting of mice almost completely prevented sensitization [119, 120]. When challenged again with the allergen, only a weak swelling response was observed. On the other hand, when administered in combination with nickel, IL-12 served as a potent adjuvant and nickel-specific swelling responses could be induced [119]. These results suggest that IL-12 is involved in the pathogenesis of contact sensitivity.

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Sequential autoantigenic determinants of the small nuclear ribonucleoprotein Sm D shared by human lupus autoantibodies and MRL *lpr/lpr* antibodies

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SUMMARY

Autoantibodies directed against the Sm proteins of the spliceosome complex are found in approximately 25% of systemic lupus erythematosus (SLE) patient sera. To determine which regions of the Sm D polypeptide are involved in the lupus autoimmune response, binding to overlapping octapeptides of Sm D has been evaluated with sera from nine Sm D-positive patients, six patients with other autoimmune serology, and five normal human sera. Lupus patient sera which are Sm precipitin-positive bind various combinations of five regions of the peptide. The major antigenic region, Epitope 5 (REAVA$(GR)_{10}$GGPRR), is bound by eight of nine Sm precipitin-positive sera tested. This region of Sm D shows significant sequence homology with Epstein–Barr nuclear antigen-1. To determine the fine specificity of the murine Sm response, four unique Sm D MoAbs derived from MRL *lpr/lpr* mice and three adult anti-Sm-positive MRL *lpr/lpr* mouse sera have been analysed. Two of these monoclonals, KSm 4 and Y12, as well as the MRL *lpr/lpr* sera tested, show binding with Epitope 5. Another of these monoclonals, KSm 2, binds octapeptides 84–91, DVEPKVKSKKREAVAG, which corresponds to Epitope 4 of this study. Antibodies from SLE patients with autoimmune serology other than anti-Sm bind the carboxyl glycine-arginine repeat $(GR)_{10}$ peptides of Sm D. However, none of the antibodies tested from patients who do not have lupus and who have different autoimmune serology binds any of the Sm D octapeptides. Normal controls did not significantly bind any of the Sm D octapeptides. These results describe two major regions of shared antigenicity of Sm D between sera from SLE patients and MRL *lpr/lpr* mice, thereby establishing a basis for the cross-species similarity of autoimmunity to the Sm autoantigen in SLE.

Key words snRNPs Sm D systemic lupus erythematosus

INTRODUCTION

Systemic lupus erythematosus (SLE) is a rheumatic disease which is characterized by a constellation of physical symptoms and serological abnormalities. Autoantibodies directed against components of the spliceosome complex are common in this disorder, and are present in sufficient concentration and complexity to form precipitins in Oüchterlony immunodiffusion. Anti-Sm precipitin autoantibodies bind ribonucleoprotein complexes containing the U1, U2, U4/U6, or U5 RNAs and have a reaction of partial identity with the anti-nRNP specificity which is composed of a ribonucleoprotein complex containing only the U1 RNA.

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Most anti-Sm sera contain antibodies directed against Sm B, Sm B′ and Sm D proteins. Some anti-Sm sera (approximately 10%) have been described as being mounted only against the Sm B/B′ peptides. No patient serum has yet been described which binds only to Sm D. Recently Sm D has been separated into two bands (D and D′) in urea containing SDS polyacrylamide gels [1] or into three bands, D1, D2, and D3, in high-TEMED gels [2]. The D1 protein has been shown to be the major target of autoimmune anti-Sm D responses, recognized strongly by 92% of Sm D-positive sera tested, and corresponds to the published Sm D sequence [3]. Sm D has three interesting domains in its carboxyl region: a lysine-rich hydrophilic area (amino acids 82–93), a $(gly\text{-}arg)_{10}$ repeat (amino acids 97–114), and a protamine-like sequence (amino acids 114–119) [3].

Regions of antigenicity of the U snRNP polypeptides have been previously determined by recombinant expression of pep-

tide fragments of Sm B/B′ [4,5], Sm D [6], nRNP A [7,8], and nRNP 70K [9–16]. Large synthetic peptides of various regions of Sm D [17], Sm B/B′ [18], and nRNP A [19] have been analysed for antigenicity. In a previous study we have constructed the overlapping octapeptides of the entire coding region of Sm B/B′ and mapped its areas of specific reactivity [20]. In that study, all Sm, nRNP precipitin-positive SLE sera tested strongly bound a repeated motif, PPPG(M,I)(R,K), which has sequence similarity with Epstein–Barr nuclear antigen-1 (EBNA-1).

In the present study overlapping octapeptides of the encoding regions of Sm D [3] were constructed by solid-phase peptide synthesis. Antigenicity of each octapeptide was determined with a variety of sera from rheumatic disease patients and normal controls.

MATERIALS AND METHODS

Sera

Human sera from patients who satisfied the classification criteria of the American Rheumatism Association for SLE [21] or normal age-matched, sex-matched controls were used in this study. Fifteen sera from patients with SLE were tested. Nine of these sera contained antibodies which formed strong precipitin lines with Sm and bound a 16-kD band in HeLa cell extract by immunoblot. Three patients formed a strong precipitin line with nRNP. Two additional patients formed a strong precipitin line with Ro and La, while another formed a strong precipitin line with Ro alone. These three patients also bound appropriate molecular weight proteins (60 kD for Ro and 45 kD for La) by immunoblot. Three patients with other autoimmune disease were also tested. These patients each had precipitating nRNP antibodies.

Four additional MRL *lpr/lpr* sera were tested for reactivity with the overlapping octapeptides of Sm D. One of these sera, collected before any evidence of an autoimmune process was evident, was from a very young littermate of the other mice. The three additional sera were from adult MRL *lpr/lpr* mice which bind Sm D, Sm B/B′, nRNP 70K, and nRNP A by immunoblot.

Monoclonal antibodies

KSm 1, KSm 2, KSm 4 and KSm 5 were derived from an MRL *lpr/lpr* mouse using hybridoma MoAb technology as described previously [22] and were generously provided by D.G. Williams (Kennedy Institute, London, UK). Supernatants containing KSm 1, KSm 2, KSm 4 and KSm 5 MoAbs were collected from cloned murine hybridoma cell lines in RPMI 1640 with 10% fetal bovine serum (FBS), glutamine, penicillin and streptomycin. These monoclonal autoantibodies were all of the IgG2a subclass. Supernatant containing Y12 MoAbs was kindly provided by Joan Steitz (Yale University, New Haven, CT).

Solid-phase peptide synthesis

The amino acid sequence of the coding region of Sm D was used to construct all of the possible overlapping octapeptides from Sm D [3] using the method previously described [20, 23–25]. These overlapping octapeptides were simultaneously synthesized at the rounded ends of radiation derivatized polyethylene pins which were arranged in the format of a 96-well microtitre plate (Coselco Mimotopes Pty Ltd, Victoria, Australia). Control pins composed of amino acids in a random sequence were prepared which were not present in any Sm or nRNP antigen sequence. In addition, positive and negative control pins were synthesized from a known reactive and non-reactive sequences of the La autoantigen.

Solid-phase cleavable peptide synthesis

Four cleavable octapeptides were constructed as previously described [26]. These peptides were then analysed for amino acid content [27] and the correct amino acids in the proper ratios were present in all of the peptides tested.

Solid-phase anti-peptide assay

Wash steps and incubations were carried out in sealed plastic containers. First, pins were blocked with 3% low-fat milk in PBS for 1 h at room temperature. Pins were then incubated in 1 : 100 dilutions of patient sera, 1 : 100 dilutions of mouse sera, or 1 : 10 dilutions of mouse ascites in 3% milk/PBS with 0·05% Tween (PBS-T) overnight at 4°C in humidified sealed containers. The pin blocks were then washed four times with PBS-T for 10 min, each with vigorous agitation. Next, each pin was incubated with anti-human γ chain-specific IgG raised in a goat, affinity purified and conjugated to alkaline phosphatase (Jackson Immunoresearch Labs, West Grove, PA) at a 1 : 10 000 dilution or with anti-mouse γ chain-specific IgG raised in a rabbit, affinity purified and conjugated to alkaline phosphatase at a 1 : 1000 dilution. Para-nitrophenyl phosphate disodium was used as a substrate for alkaline phosphatase, and plates were read at 405 nm with a MicroELISA Reader (Dynatech, Alexandria, VA). Results for each plate were then standardized by comparison with positive control pins.

After completion of an assay, pins were sonicated for 2 h in sonication buffer (40 g SDS, 4 ml β-mercaptoethanol, and 62·4 g sodium phosphate to 4 *l*) to remove antibodies, conjugate and substrate. After sonication pins were washed twice in hot water and boiled in methanol for 2 min. Pins were then allowed to air dry for a minimum of 10 min and stored with desiccant or used for another assay.

Anti-$(GR)_6$ absorption

The MAP $(GR)_6$ peptide was covalently bound to cyanogen-preactivated Sepharose 4B by the method recommended by the manufacturer [28,29]. Fifteen milligrams of the $(GR)_6$ peptide were added per gram of Sepharose 4B used, sufficient to prepare a 2-ml column. One millilitre of each human lupus serum tested was circulated through this column for a total of four passes at 4°C overnight and constituted the absorbed serum. More than 90% of anti-$(GR)_6$ reactivity was removed as measured by standard ELISA.

ELISA

Standard solid-phase assays were used to measure the antibody reactivity in normal and lupus sera before and after absorption of the anti-MAP $(GR)_6$ anti-peptide antibodies. One microgram of antigen (Sm, nRNP, or MAP $(GR)_6$) was coated per well in each of 96 polystyrene wells in 125 μl of coating buffer (Na_2CO_3 34·98 g, $NaHCO_3$ 64·46 g to 2200 ml distilled water pH 9·6) for 2 h at room temperature or at 4°C overnight. Wells were then washed once with 250 μl of PBS-T (NaH_2PO_4 0·2 M, NaH_2PO_4 0·2 M, NaCl 1·5 M, and 0·05% Tween pH 7·4) per well. Wells were blocked with 150 μl of 0·1% bovine serum albumin (BSA) in PBS per well for 2 h at room temperature. After washing the plates, sera (human control serum or lupus patient serum with

and without anti-$(GR)_6$ antibodies) at varying dilutions were added to each well and plates were allowed to incubate for 2 h at room temperature or overnight at 4°C. After incubation, plates were washed four times with 250 μl of PBS-T/well. Next, each well was incubated for either 2 h at room temperature or overnight at 4°C with anti-human γ chain-specific IgG raised in a goat, affinity purified and conjugated to alkaline phosphatase (Jackson Immunoresearch Labs) at a 1:10000 dilution. Para-nitrophenyl phosphate disodium was used as a substrate for alkaline phosphatase and plates were read at 405 nm with a MicroELISA Reader (Dynatech).

Western blot

Affinity-purified Sm and nRNP antigens and HeLa cell extract were subjected to electrophoresis in 12·5% polyacrylamide gels containing 1% SDS (in 0·15 M Tris HCl, pH 8·8) using a protocol described previously [30]. Human lupus sera (with and without absorption of anti-$(GR)_6$ antibodies) and human controls were tested for reactivity with the Sm and nRNP proteins.

RESULTS

Nine anti-Sm-positive SLE sera have been analysed for reactivity with overlapping octapeptides which span Sm D. Each of the nine patient sera bound octapeptides derived from Sm D. The binding pattern of a representative patient serum is presented in Fig. 1c.

Anti-Sm D sera from patients with SLE shared reactivity with five different regions of Sm D. Each of the other anti-Sm patient sera tested had very similar reactivity to the representative serum presented in Fig. 1. Not surprisingly, therefore, the aggregate binding of these nine sera, showed as a cumulative average reactivity in Fig. 2a, closely resembles the representative serum in Fig. 1c.

For purposes of this study a common epitope is defined as having an average reactivity >2 s.d. above the normal mean and being bound by at least three of the patient sera. There are five regions of Sm D that appear to be antigenic by this definition (Table 1).

Five normal human sera have shown minimal reactivity with no specific regions of antigenicity demonstrated with the overlapping octapeptides of Sm D (Fig. 1b and Fig. 2b). These octapeptides of Sm D have also been screened for non-specific, background binding to anti-human conjugate alone (Fig. 1a). Very little background reactivity has been demonstrated in either group of controls.

The shared antigenic regions bound by each anti-Sm precipitin-positive serum tested are presented in rows 1–9 of Fig. 3. Epitope 4, octapeptides 82–90, is bound by seven of nine sera and exhibits the highest average binding of the Sm D peptides, 6 s.d. above the normal mean. Eight of nine anti-Sm D SLE patients are reactive with the sequence spanning octapeptides 92–112, Epitope 5, with an average binding of 4 s.d. above the normal mean. Epitope 2 also has average binding 3 s.d. above the normal mean, and is reactive with seven of nine anti-Sm D sera tested. Epitope 1 and Epitope 3 are recognized by fewer sera, five and three respectively, and have average binding 2 s.d. above the normal mean.

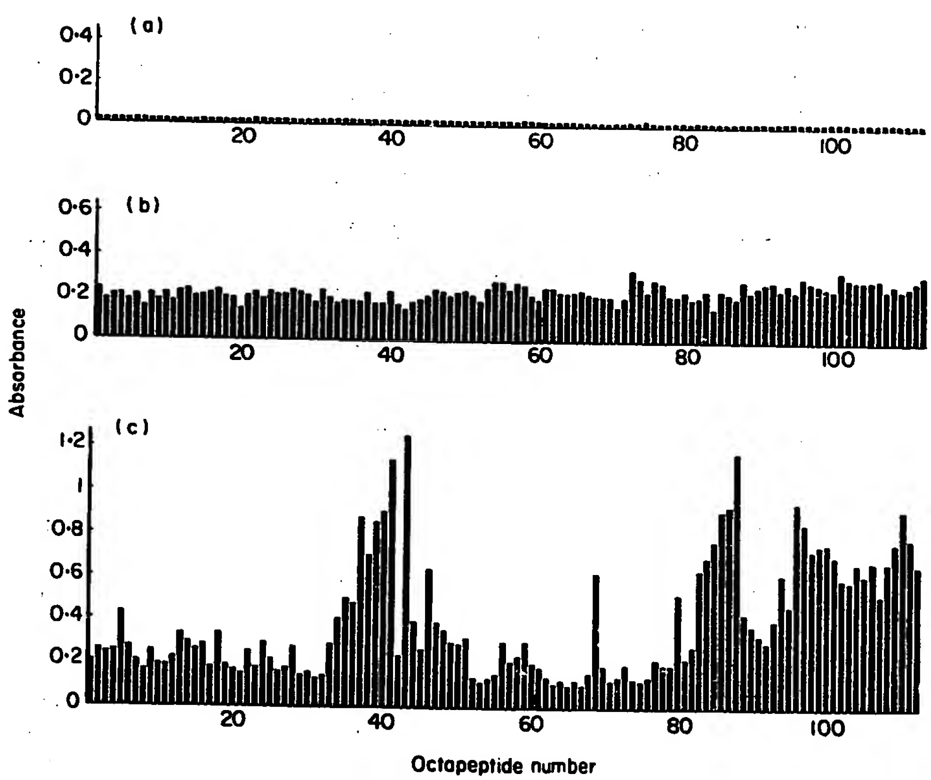

Fig. 1. Antigenicity of overlapping octapeptides of the Sm D-associated protein. (a) Background reactivity of the octapeptides with anti-human IgG conjugate alone. (B) Reactivity of the octapeptides with a normal human serum. (C) Binding of a representative anti-Sm precipitin-positive systemic lupus erythematosus patient serum.

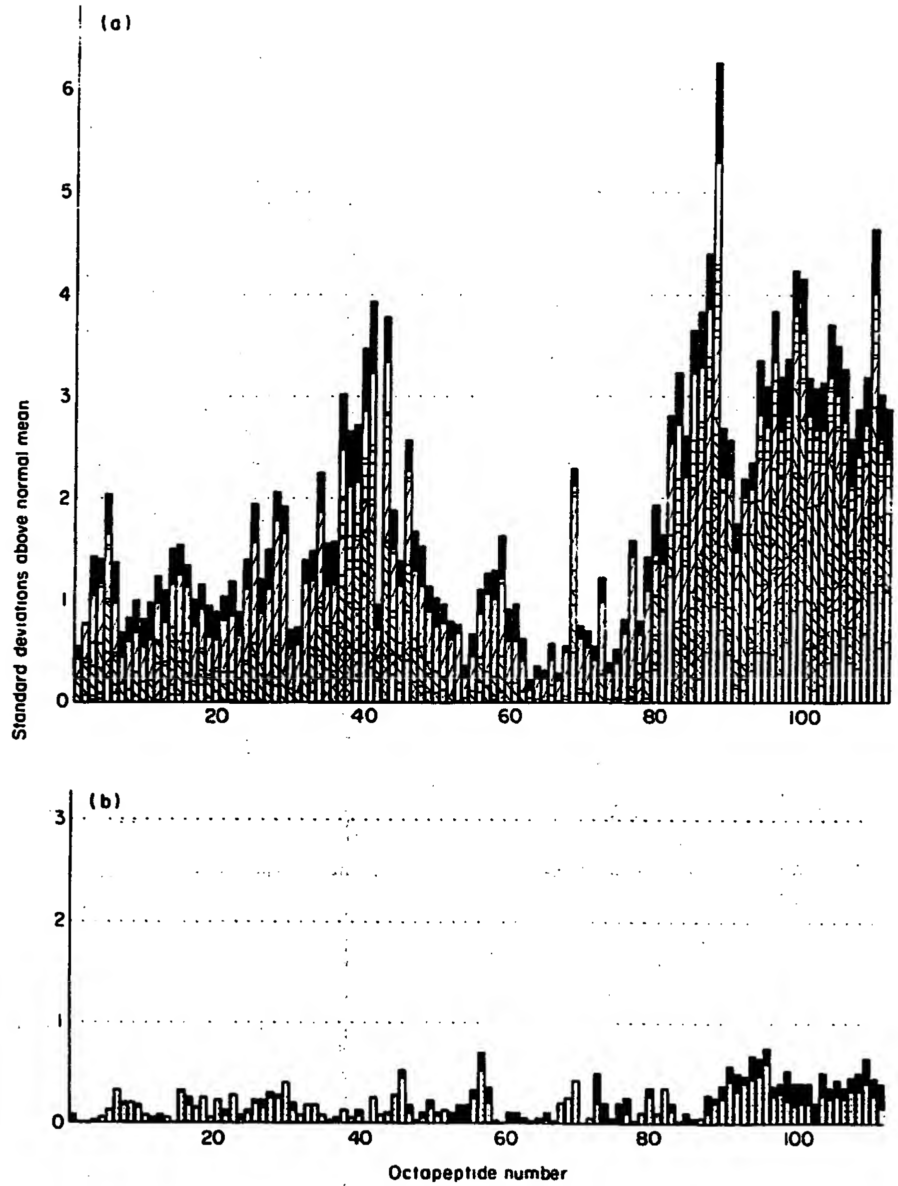

Fig. 2. Mean binding to overlapping octapeptides by anti-Sm precipitin-positive patient sera. (a) Contribution of each anti-Sm D lupus patient to the average reactivity of anti-Sm patient sera. (b) Contribution of each normal to the average reactivity of normal sera. Data are expressed as the s.d. above the mean of the normal control sera for each octapeptide. The s.d. has been calculated from the binding of all controls for all of the Sm D octapeptides, evaluated as a group. The contribution of each patient and normal serum is represented by different patterns in the histogram.

Six lupus patients with autoimmune serology other than anti-Sm have also been tested for reactivity with these peptides of Sm D (rows 10–15 of Fig. 3). All six of these patient sera bind the carboxyl terminal $(GR)_{10}$ of Epitope 5. Five of six of these SLE sera have antibodies which react with Epitope 2 and five of six sera react with Epitope 4.

Three additional anti-nRNP sera, from patients with autoimmune rheumatic diseases other than SLE, have also been tested. None of these sera bind to any of the peptides by > 2 s.d. above the normal mean.

To assess for the specificity of the binding of the anti-Epitope 5 antibodies to the Sm proteins, anti-$(GR)_6$ antibodies were obtained by affinity purification. Three anti-Sm and anti-nRNP lupus patient sera (patients 1, 2 and 9 of Fig. 3), as well as three anti-nRNP alone patient sera (patients 10, 11 and 14), were passed over a $(GR)_6$ peptide column. Antibodies eluted from this column all bound the $(GR)_6$ peptide, in addition to purified Sm antigen, as detected by ELISA. The eluted antibodies from patient 1 were tested on the 112 Sm D octapeptides. They bound Epitope 5 of Sm D without binding octapeptides from other regions of Sm D.

The specific binding activity against both antigens (Sm and MAP $(GR)_6$) was increased by more than four- to seven-fold in the MAP $(GR)_6$ eluate from every anti-Sm precipitin-positive serum tested. Anti-$(GR)_6$ antibodies collected from the anti-nRNP precipitin-positive and anti-Sm precipitin-negative

Table 1. Amino acid sequences of linear antigenic regions of the Sm D antigen

Sm D epitopes
Epitope 1: RFLMKLSH (5)
Epitope 2: NTHLKAVKMTLKNR (37–43) and TLKNREPV (46)
Epitope 3: ILPDSLPL (69)
Epitope 4: DVEPKVKSKKREAVAG (82–90)
Epitope 5: REAVA$(GR)_{10}$GGPRR (92–112)

Antigenic regions are defined as an octapeptide with an average binding by the nine anti-Sm precipitin-positive patients of ≥ 2 s.d. above the mean of normal sera and found in at least three of the nine patients. The positions of the amino terminal amino acid of each octapeptide of the antigenic region is given in parentheses.

	1 (5)	2 (37–43, 46)	3 (69)	4 (82–90)	5 (92–112)
1	+	+	+	+++++	+++++
2	+	+++++	+	+++++	+++
3	–	–	–	–	+
4	+	+	–	+++	+
5	–	–	–	–	+++++
6	+	+++	+++	+++++	+
7	–	+	–	+	+
8	–	+++	–	+++++	–
9	+	+++	–	+++++	+++
10	–	+	–	+	+
11	–	+	–	+	+++++
12	+	+	–	+	+
13	+	+	+	+++	+
14	–	+	+	++++	++++
15	–	–	+	–	+
K Sm 1	–	–	–	–	–
K Sm 2	–	–	–	+	–
K Sm 4	–	–	–	–	+
Y12	–	–	–	+	+
lpr/lpr 1	–	–	–	–	–
2	–	–	–	–	+
3	–	–	–	–	+

Fig. 3. Common epitopes of Sm D bound by systemic lupus erythematosus patients. Anti-Sm precipitin-positive lupus patients are presented in rows 1–9. Lupus patients with other autoimmune serology are presented in rows 10–15. Murine MoAbs are listed in the next four rows. The last three rows present the binding patterns of MRL *lpr/lpr* sera including a young littermate with no detectable autoantibodies (*lpr/lpr* 1), a mature MRL *lpr/lpr* mouse (*lpr/lpr* 2), and a pool of two other adult MRL *lpr/lpr* mice (*lpr/lpr* 3). The columns represent specific antigenic regions of Sm D. Patient reactivity is determined as binding by patient sera in excess of 2 s.d. above the normal mean (+) with each 2 s.d. of reactivity also indicated (+).

patient sera bound Sm at a much lower titre than did those patient sera containing both anti-Sm and anti-nRNP precipitins. Nevertheless, the anti-MAP $(GR)_6$ eluate from the anti-nRNP precipitin-positive patient sera tested had a three- to four-fold increase in anti-Sm binding compared with the pre-absorption anti-nRNP patient sera from which they had been obtained.

Also, a significant proportion of the anti-Sm response was absorbed by MAP $(GR)_6$. This absorption reduced the binding to Sm by 36% in patient 1, 11% in patient 2, and 46% in patient 9. The antibodies eluted from MAP $(GR)_6$ from these sera also bound Sm D in Western blots of both HeLa cell extract and purified Sm or nRNP autoantigen.

Anti-Sm D murine MoAbs, KSm 1, KSm 2, KSm 4 and Y12, have been tested for binding with the overlapping octapeptides of Sm D. KSm 2 specifically bound one region of Sm D which spans octapeptides 84–91 (Fig. 4a). This region corresponds to the Epitope 4 recognized by many SLE patients. KSm 4 bound to Epitope 5 and also had additional binding to octapeptide 79 (Fig. 4b). Y12, on the other hand, bound both Epitope 4 and Epitope 5 (Fig. 4c). Octapeptides 95–111, which is a nearly complete subset of Epitope 5, were bound by two of these MoAbs and by the sera from mature *lpr/lpr* mice. This is a major area of reactivity in both human and mouse sera. Neither KSm 1 nor an anti-Sm B/B′-specific MoAb, KSm 5, bound any of the overlapping Sm D octapeptides.

In addition to murine MoAbs, MRL *lpr/lpr* sera containing spontaneously arising autoantibodies have been screened for reactivity with different regions of Sm D. Mature MRL *lpr/lpr* mouse sera, which immunoblot Sm and nRNP proteins, display considerable reactivity with the human lupus Epitope 5 (Fig. 5b,c). This murine response is directed specifically against the $(GR)_{10}$ repeat spanning amino acids 96–110. Curiously, the RGRGRGRG sequences were bound more avidly by the murine sera than to their neighbours the GRGRGRGR sequences. A pre-autoimmune MRL *lpr/lpr* serum (immunoblot negative) showed no reactivity with any of the octapeptides of Sm D (Fig. 5a).

DISCUSSION

Five epitopes of the Sm D autoantigen have been described. Nearly all of the anti-Sm sera tested bound Epitopes 2, 4 and 5, thereby defining the major linear antigenic regions of this common lupus autoantigen.

All anti-Sm D MoAbs and MRL *lpr/lpr* autoantibodies tested appeared to be entirely directed against Epitope 4 and Epitope 5, which are also the major targets of the human anti-Sm response in SLE. The KSm 2 murine MoAb bound Epitope 4, the most reactive portion of Sm D in this study. Two additional murine monoclonals, KSm 4 and Y12, were reactive with the carboxyl region, Epitope 5, which was bound by virtually all (89%) of the human lupus sera tested. In addition, Y12 was also reactive with Epitope 4. The MRL *lpr/lpr* sera have antibodies predominantly directed against the carboxyl terminal Epitope 5. Astonishing similarity in the binding to Sm D octapeptides is found when the anti-Sm D autoimmune responses of human and murine lupus sera are compared.

In a previous study of the fine specificity of the Sm B/B′ response in lupus patients [20], we appreciated that Sm precipitin-positive sera contain antibodies which cross-react with a sequence, PPPGRRP, in EBNA-1. No normal controls, who were all EBV antibody-positive, bound this region; however, all of the anti-Sm D immunoblot-positive sera evaluated in this study bound this region of EBNA-1 (data not presented). In addition, Epitope 5 of Sm D contains a glycine-arginine repeat

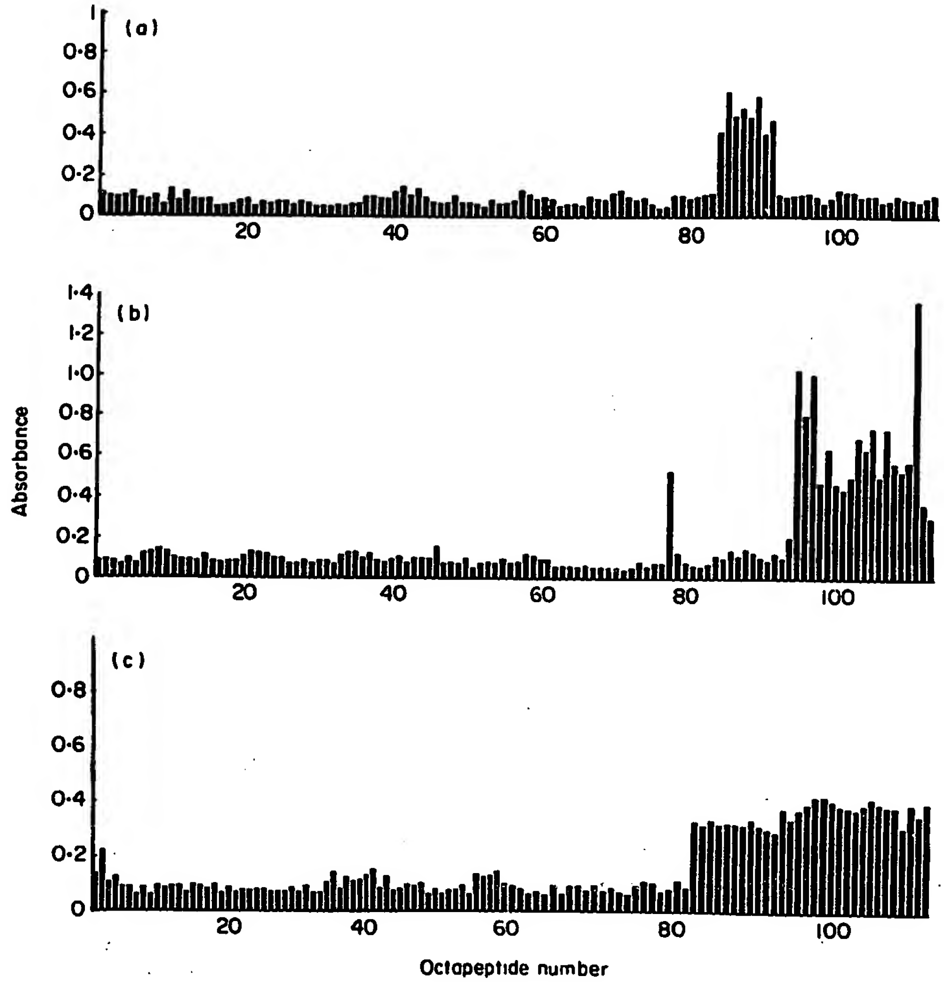

Fig. 4. Sm D octapeptides bound by three anti-Sm MoAbs including KSm 2 (a), KSm 4 (b) and Y12 (c). KSm 2 and KSm 4 were generous gifts from D. G. Williams. Y12 was graciously provided by Joan Steitz.

of which shorter versions are found in EBNA-1. $(GR)_{10}$ is also bound by lupus patient sera, but not with normal controls. These data suggest that lupus patients and normal humans may mount different immune responses against EBV.

This study has defined in detail the linear antigenic regions of Sm D. This work has shown that from 11% to 46% of the anti-Sm response may be directed against one short region of this Sm protein (or against this linear region which is brought together with other antigenic regions on the native surface of this spliceosomal protein). In one other system, 40–85% of the entire anti-nRNP autoantibodies' response is directed against two linear antigenic regions of nRNP A in a subset of patients (J. James & J. Harley, unpublished data). The crystallized structure of this portion of nRNP A is known, and these two linear epitopes are present on the contiguous surface of this autoantigen.

SLE patient sera with autoantibodies other than anti-Sm by immunoprecipitation (i.e. anti-Ro, anti-La and/or anti-nRNP) also bind the major epitopes of Sm D. These patient sera consistently bind to the $(GR)_{10}$ repeat of Epitope 5. Rheumatic disease patients (not including patients with SLE) with these same autoantibodies (i.e. anti-nRNP), however, do not bind any regions of Sm D. The most interesting hypothesis to be drawn from these results is that the common binding of lupus patient sera to these specific octapeptides may reflect a common immunopathogenic mechanism. Additional experiments are warranted to address these issues.

Barakat *et al.* tested patient reactivity with seven synthetic peptides [18]. Three peptides were found to be reactive with 67%, 89% and 33% of anti-Sm lupus patients, respectively (top line, Fig. 6). The first reactive regions spanned amino acids 1–20 and correspond to our Epitope 1. Amino acids 44–67 are included in the second reactive region and contain our Epitope 2. Epitope 5 of this study is included in the third reactive region described by Barakat *et al.* In addition to establishing the fine specificity of Sm D-derived peptide binding, our work confirms the presence of epitopes in the regions of the molecule identified by Barakat *et al.* [18] using a fundamentally different technological approach.

In a recent article, Rokeach *et al.* described binding to a set of seven TrpE fusions of various regions of Sm D [27]. They identified two sets of anti-Sm sera: one group which recognizes the carboxyl terminus and another group that only binds the near full-length Sm D recombinant polypeptide. The most frequently recognized fragment spanned amino acids 87–119 and was bound to some degree by 13 of 19 anti-Sm sera as tested by immunoblot. This region corresponds to our Epitope 4 and Epitope 5 (Fig. 6).

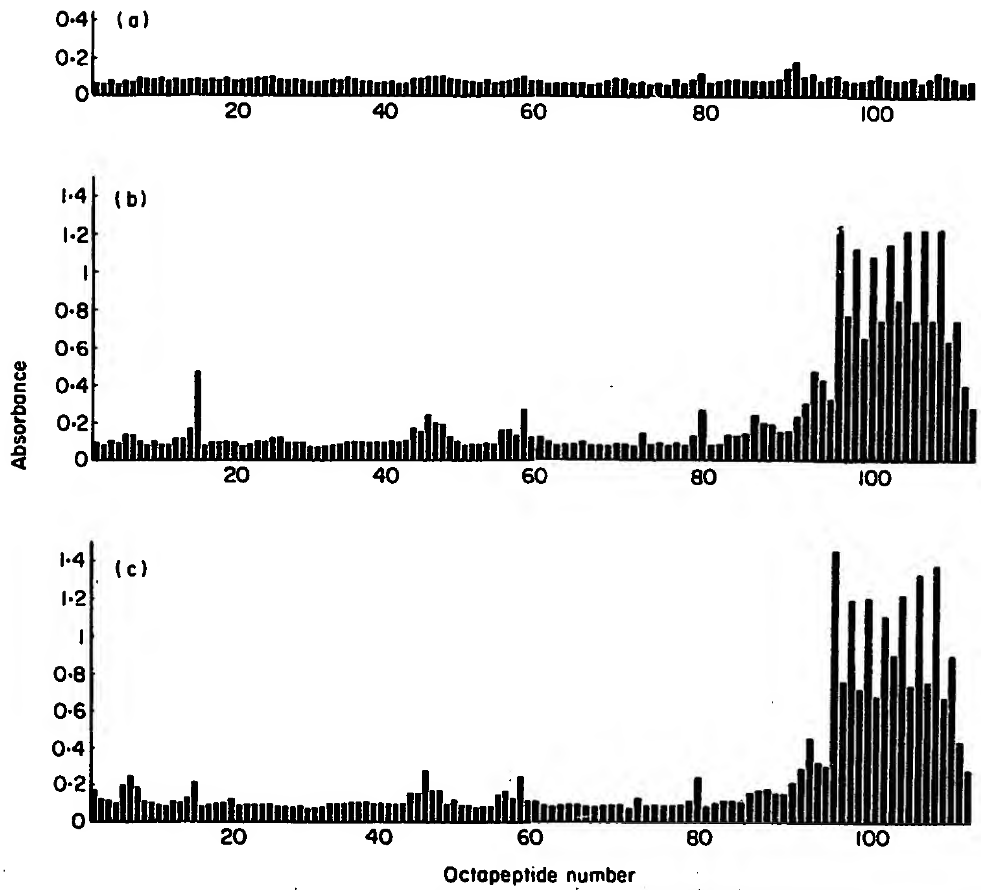

Fig. 5. Antibody binding patterns of MRL *lpr/lpr* sera to Sm D octapeptides. (a) Minimal binding of an immature MRL *lpr/lpr* littermate of the mice presented in the other two panels. (b) Reactivity of serum from a mature MRL *lpr/lpr* mouse. (c) Antigenicity of these peptides with a pool of two MRL *lpr/lpr* sera from mature mice.

Autoantibodies are clearly involved in the pathogenesis of SLE. However, the inciting event which triggers the anti-Sm response in SLE is still unknown. Defining the molecular structures of the autoantigen that interact with autoantibody may be very important in coming to understand autoimmunity. A subset of these structures is likely to be important in discovering not only the origin of the observed humoral autoimmunity but also the molecular mechanism that induces such a profoundly aberrant immune response. Identification of these sequences has already led to the realization of an unlikely homology of antigenic sequences with regions of EBNA-1. Hopefully, further analysis of the autoimmune process in these patients will lead to a valid hypothesis for the etiology and pathogenesis of SLE.

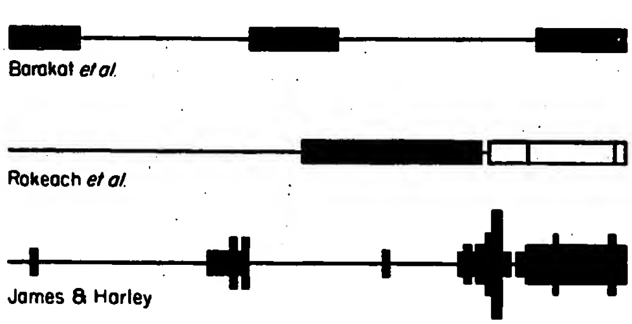

Fig. 6. Comparison of epitopes previously described for Sm D with the results of this work. The top line demonstrates epitopes mapped by Barakat *et al.* [18]. The next line shows antigenic regions presented by Rokeach *et al.* [27]. The last line presents the linear autoantigenic determinants described in this work. Wider bars denote greater average reactivity of serum from patients with anti-Sm precipitin-positive systemic lupus erythematosus.

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Alterations in the structure of the EBV nuclear antigen, EBNA1, in epithelial cell tumours

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The EBV nuclear antigen, EBNA1, is the only viral protein consistently expressed in all virus-infected cells. It is required *in trans* for viral replication, maintenance of EBV extrachromosomal episomes, and transcriptional transactivation in latently-infected B-cells. It binds RNA suggestive of a regulatory role in post-transcriptional events and in transgenic mice, it is tumorigenic. In RNase protection studies relating to the EBV-associated tumour, nasopharyngeal carcinoma (NPC), we show that a C-terminal EBNA1 RNA probe from the prototype B95-8 marmoset strain can protect its own mRNA from enzymatic digestion, but does not fully protect EBNA1 mRNA from NPC cells. This finding is consistent with changes in the coding region for the antigen. We thus determined the sequences of EBNA1 genes derived from an NPC xenograft and numerous patient biopsies and identified a number of mutations in the gene in these human cells, relative to B95-8. Many of the nucleotide changes would lead to non-conservative amino acid alterations in apparently functionally significant regions of the protein. We show that although some of the mutations lie in regions designated as critical to DNA binding, they have negligible effect on this property of EBNA1. The basic regions in EBNA1 that may bind to RNA, at least *in vitro*, are exempt from mutation. Thus, unless the alterations are 'silent', which for such a critical viral function seems unlikely, they may relate to as yet unmapped viral activities, such as a role in tumorigenesis and the ability of EBNA1 to evade the cellular immune system, or be associated with the ability of the antigen to regulate gene transcription.

Keywords: nasopharyngeal carcinoma; mutations; DNA binding; Epstein-Barr virus

Introduction

One of the key functions of the oncogenic human herpes virus, Epstein-Barr virus (EBV), is the nuclear antigen, EBNA1, the open reading frame for which lies in the *Bam*HI K region of the viral genome (Baer *et al.*, 1984). In all cells carrying the virus, whether established B-cell lines or tumours, EBNA1 is expressed. In different cell lines sizes vary between about 69–96 kD depending upon the size of the large internal glycine/alanine repeat sequence the protein accommodates. The 88 kD EBNA1 expressed in latently infected IB4 cells, generated with virus from B95-8 cells, has been identified as a phosphoprotein, containing phosphoserine (but not apparently phosphothreonine or tyrosine) residues (Hearing and Levine, 1985). EBNA1 plays a role in B-cells in maintenance of the viral episome and, as far as is known, is the only viral protein necessary for replication of the latent form of EBV (Yates *et al.*, 1984, 1985; Lupton and Levine, 1985; Reisman and Sugden, 1986). These functions are mediated by strong binding of the antigen to DNA at two sites (designated I and II), within *ori*P, in the *Bam*HI C region of the genome (Reisman and Sugden, 1986; Chittenden *et al.*, 1989); weaker binding occurs at a third site (III) in *Bam*HI Q (Jones *et al.*, 1989; Ambinder *et al.*, 1990). A 'core' DNA binding motif has been identified within EBNA1 (Chen *et al.*, 1994). The transcription of EBNA1 is complex, three different promoters having been identified [in *Bam*HI fragments C, W and F in different cellular settings (reviewed, Speck and Strominger, 1989; see also Sample *et al.*, 1991; Schaeffer *et al.*, 1991; Smith and Griffin, 1992)]. Whereas the F-promoter (Fp) is functional in some B lymphocytes and epithelial cells, there is no evidence that either Cp or Wp are active in the latter. EBNA1 may also act as a transcriptional enhancer (Yates and Camiolo, 1988; Sugden and Warren, 1989; Ambinder *et al.*, 1990) or exert a repressor function, regulating its own production; the latter is mediated by binding to sequence within *Bam*HI Q (Sample *et al.*, 1992). We have shown that EBNA1 can bind to RNA, including a region encoded within its own transcript, at least *in vitro* using apparently 'RGG' binding motifs, three of which exist within the protein (Kiledjian and Dreyfuss, 1992; Snudden *et al.*, 1994). The EBNA1 gene (from B95-8 cells), introduced into transgenic mice, can lead to tumour formation (Wilson and Levine, 1992). One of the more perplexing problems associated with this antigen is the ability of EBNA1 expressing cells to escape the cellular immune system (Khanna *et al.*, 1992; Murray *et al.*, 1992; Trivedi *et al.*, 1994). Recently it has been reported that antisense oligodeoxynucleotides to EBNA1 can inhibit proliferation of EBV-immortalized B cells (Roth *et al.*, 1994). Thus, in view of the importance of EBNA1 to the biology of EBV infected cells, it seems reasonable to expect constraints to exist on the sequence of this gene, in particular within the open reading frame and in the genomic sites to which EBNA1 binds.

In recent years we have been examining EBV gene expression in epithelial cells noting that expression in NPCs, as defined by study of the human NPC tumour C15 which is passaged in nude mice, often differs from that identified in B-cells (Hitt *et al.*, 1989; Smith and Griffin, 1991, 1992). Other experimentors have pointed to viral gene alterations that seem specific for NPCs (Lung *et al.*, 1990; Hu *et al.*, 1991; Chen *et al.*, 1992).

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However, due to the crucial roles played by EBNA1, as cited, we were surprised to find that in ribonuclease protection assays, a viral probe from one population of cells (marmoset B95-8) whilst protecting the EBNA1 message from the same cells, failed to provide full protection for the corresponding mRNA from several other EBV-infected cell types. These data were indicative of sequence alterations and seemed of particular relevance in that extensive sequencing of another region – the *Bam*HI I and A regions of the C15 viral genome (Hitt *et al.*, 1989; Smith *et al.*, 1993) – had revealed few base changes. These observations have led us to investigate alterations which might exist throughout the EBNA1 coding sequence, to determine whether, on the one hand, they were largely confined to 'wobble' positions in coding triplets (thus being essentially silent), to conservative amino acids or (possibly) non-functional domains of the antigen or, on the other hand, whether they might prove of functional relevance, for example, being either cell- or species-type specific. In particular, we have analysed the EBNA1 sequence in the C15 genome and in a number of primary nasopharyngeal carcinomas, as compared with the EBNA1 open reading frame in B95-8 cells. We have asked whether the alterations identified relate to mapped functions of the antigen, and observing that many of them lie within the known DNA binding region, have investigated whether they have an effect on the ability of the protein to bind to its own DNA and thus influence replication, or whether they need to be examined in the light of other known, unmapped or unidentified functions.

Results

RNase protection

RNase protection analysis of the C terminal region of EBNA1 (Figure 1) showed that a 394 bp radiolabelled RNA probe from the B95-8 *Bam*HI K fragment, within the coding sequence, gave a different protection pattern when hybridised to B95-8 (self) mRNA (Figure 1, track A) or polyadenylated RNA from C15 (Figure 1, track B) or NAD-C15 (Figure 1, track C). Whereas the full-length fragment was completely protected in the case of B95-8, four major bands, with sizes ranging from about 90 to 280 bp, were observed with C15 and NAD-C15 mRNAs. This suggested that base changes (mutations) exist in the C terminus of the NPC-derived EBNA1 gene, relative to B95-8, and prompted us to perform sequence analysis of NPC biopsies to confirm this finding.

PCR/Sequence analysis of the EBNA1 open reading frame from C15 and NPC biopsies

In order to investigate the nucleotide changes in EBNA1 in the C15 viral strain, relative to B95-8 EBNA1, and relate this to NPC biopsies, the open reading frames (ORF) from a number of sources (see Materials and methods) was sequenced. For this study, the relevant DNA was amplified by PCR using a series of oligonucleotide primers (see methods), then cloned directly into M13 for sequence analysis. The DNA encoding the long glycine/alanine repeat region in EBNA1 was omitted because of the difficulty of

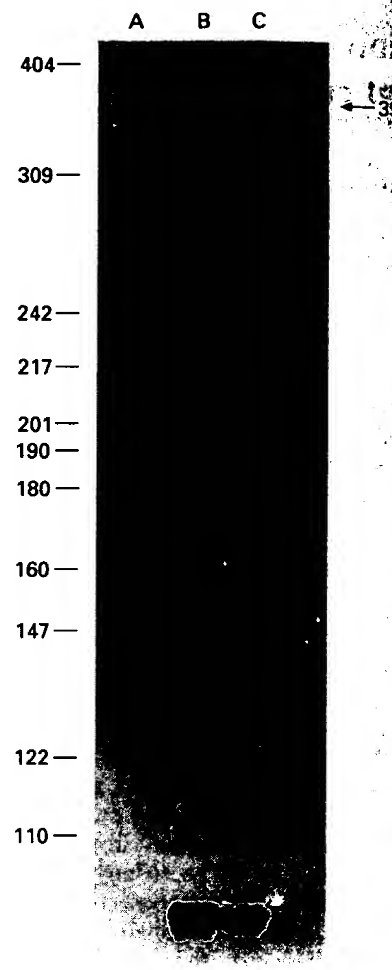

Figure 1 RNase protection analysis of the C terminus of EBNA1 mRNAs. B95-8 (track A), C15 (track B) and NAD-C15 (track C) mRNAs were hybridized with a ^{32}P-labelled RNA probe from the C-terminal region of EBNA1 (positions 109410–109804), transcribed in an antisence direction, and then digested with RNase A/T1. Digestion products were separated on a 7.5% denaturing gel. The full-length protected fragment (394 bp) is noted (arrow). Sizes of molecular weight markers are indicated

amplifying this repetitive region. Primary sequencing identified a number of base changes in the NPC and C15 DNA clones, relative to B95-8, some of which would be accompanied by non-conservative amino acid substitutions in the antigen (see Figure 2). All the NPC biopsy samples (except No. 58) had identical sequence alterations which differed slightly from those found in the C15 (or NAD-C15) tumour. No. 58 had additional nucleotide changes at aa 427 and 585, the latter resulting in a threonine to isoleucine (T to I) conversion. As the B95-8 sequence determined in this study was identical to that previously published (Baer *et al.*, 1984), changes observed in the tumour materials, confirmed by sequencing more than one clone per sample, seem unlikely to be due to errors made by Taq polymerase during the amplification process.

Analysis of the effect of EBNA1 sequence alterations on DNA binding

Numerous studies have been directed at mapping the functional domains of EBNA1 (summarised in Figure

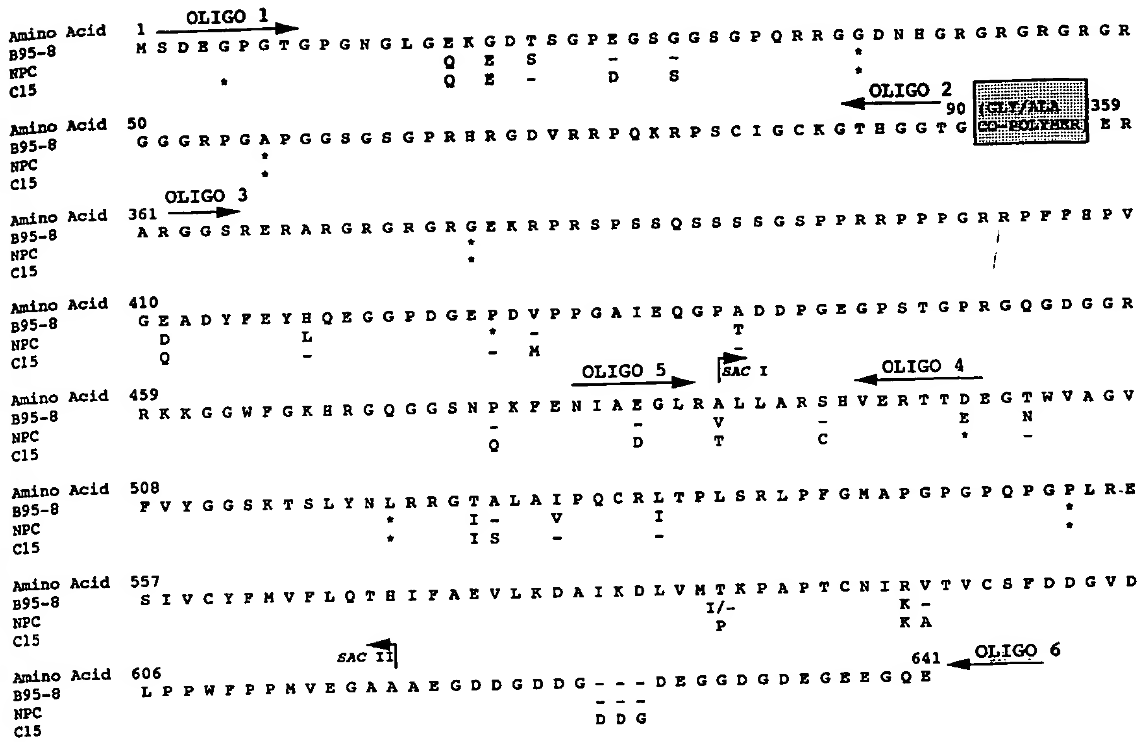

Figure 2 The amino acid changes observed by sequence analysis of C15 and NPC biopsy samples, relative to the B95-8 EBNA1 sequence. The single letter amino acid code is used. The alterations found in NPC biopsy samples and C15 are shown below the amino acid sequence derived from the BamHI K open reading frame in B95-8 EBV cells (Baer *et al.*, 1984). For specific nucleotide changes, see Table 1. A change in nucleotide sequence that does not result in an amino acid change is indicated (*); a dash (–) at a particular site indicates no change relative to the B95-8 sequence. The position of the oligonucleotides used in the PCR (see Materials and methods for details) are shown together with the position of the B95-8 repetitive (including the glycine/alanine repeat) sequence (amino acids 91–358). One NPC biopsy (No. 61) was not sequenced beyond aa 493. The location of the *Sac*I/*Sac*II subfragment from the C-terminus of the EBNA1 ORF, used in RNase protection experiments (Figure 1), is indicated

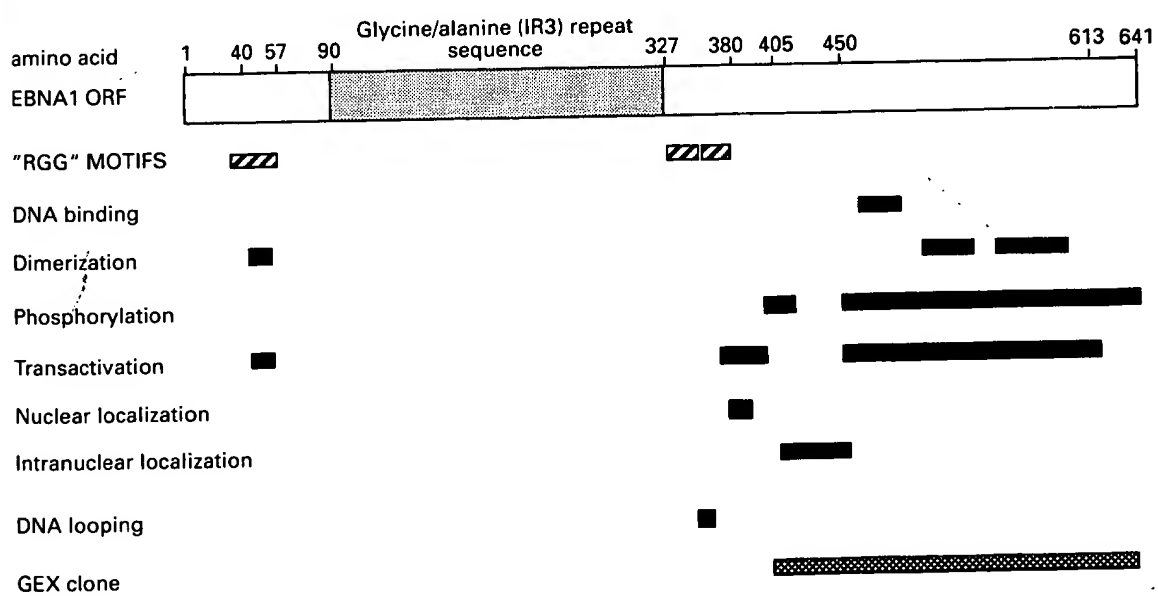

Figure 3 Schematic representation of the functional domains of EBNA1 ORF. (Adapted from Polvino-Bodnar and Schaffer, 1992.) The mapped functional domains are shown as solid horizontal bars below the conventional three domain representation of EBNA1. Data are derived from Polvino-Bodnar *et al.* (1988); Yates and Camiolo (1988); Rawlins *et al.* (1985); Polvino-Bodnar and Schaffer (1992); and Ambinder *et al.* (1991). The hatched boxes show locations of 'RGG' motifs thought to be involved in the binding of EBNA1 to RNA (Snudden *et al.*, 1994). A potential leucine zipper dimerization motif lies between amino acids 553–581 and a highly negatively charged region (17/41 amino acids) is found at the extreme C-terminus (Ambinder *et al.*, 1991). Inoue *et al.* (1991) identify a potential basic–helix–loop–helix (B-HLH) region between amino acids 467–587, possibly involved in transcriptional regulation. The region of EBNA1 DNA cloned into GEX, expressed as a fusion protein and used in DNA binding studies (Figure 6) is indicated (cross hatched)

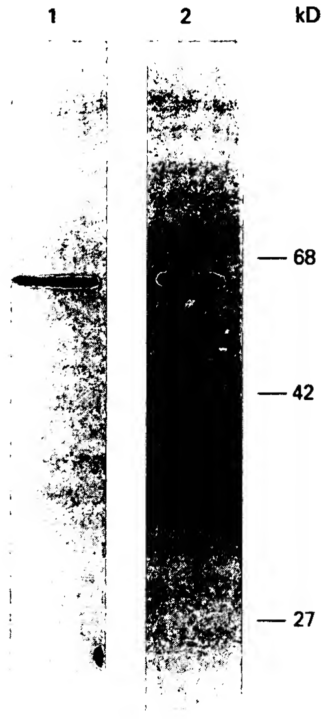

Figure 4 Synthesis and purification of EBNA1 fusion protein. Lane 1 contains a sample of purified EBNA1 recombinant protein from C15 separated on a 12.5% SDS polyacrylamide gel and stained with Coomassie blue. Lane 2 contains an immunoblot of the material used in lane 1. Positions of size markers (kD) are shown on the right

```
9021                  4                       3
TAACCCTAATTCGATAGCATATGCTTCCCGTTGGGTAACATATGCTATTGAATTAGGGTT
                                           C

                      2                       1              9134
AGTCTGGATAGTATATACTACTACCCGGGAAGCATATGCTACCCGTTTAGGGTTGGGATCC
               T
```

Figure 5 The region of dyad symmetry (region II) in ori P (adapted from Rawlins *et al.*, 1985). Region II is composed of four consensus EBNA1 binding sites. The nucleotide changes (A to T and A to C, in sites two and three, respectively) observed in C15 are indicated (bold type)

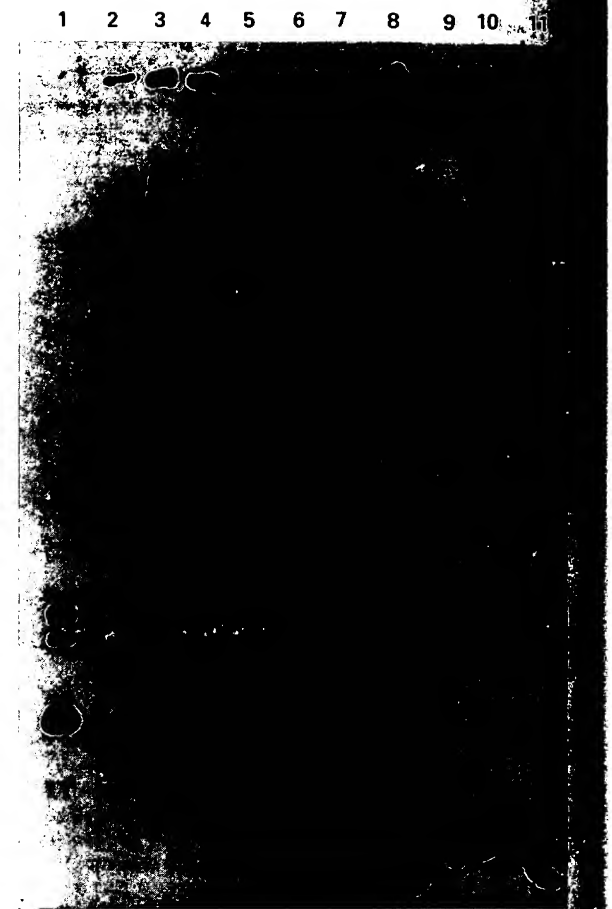

Figure 6 Binding of B95-8 and C15 EBNA1 fusion proteins to their respective region II DNAs. DNA from *Bam*HI C region II (Figure 5) was end-labelled with [^{32}P]phosphate, then 1×10^4 c.p.m. incubated with 0, 2.6 and 5.2 μg of either B95-8- (lanes 1–3) or C15-EBNA1 glutathione S-transferase fusion protein (lanes 4–6), respectively. As controls, 5.2 μg of glutathione S-transferase was incubated with B95-8 and C15 region II DNAs (lanes 7 and 8, respectively). Additionally, no protein (lane 9) or 5.2 μg of B95-8-(lane 10) or C15-EBNA1 fusion protein (lane 11) was incubated with end-labelled DNA from the *Sac*I/*Sac*II fragment from the C terminus of the EBNA1 ORF (see Figure 2). After incubation at 30°C for 20 min, complexes were separated by electrophoresis on a 4% native acrylamide gel

3). Some of the amino acid alterations identified in EBNA1 from C15 and NPC lie within these regions. In order to determine whether the changes have an effect on function, and since function is integrally linked to DNA binding, we investigated the ability of C15 and B95-8 EBNA1 polypeptides to bind DNA. The C-termini of the EBNA1 ORF from these sources were cloned into the GEX plasmid (see Figure 3) and expressed in *E. coli* as a fusion product which contains a 27.5 kD glutathione S transferase and a 250 amino acid domain of EBNA1, incorporating the 191 amino acid EBNA1 component described by Rawlins *et al.* (1985). The predicted size of the fusion protein is 58 kD. After purification to near homogeneity by affinity chromatography on immobilised glutathione, a 58 kD protein was seen on staining with Coomassie blue dye (Figure 4, lane 1). This protein was not seen in bacteria transfected with native GEX plasmid (data not shown). The identity of the fusion protein was established by immunoblotting using polyclonal antibodies to EBNA1 (Figure 4, lane 2). The polyclonal antibody also reacted with a second band of about 37 kD, a degradation product of EBNA1, just visible on staining with Coomassie blue.

To assess DNA binding, a gel mobility retardation assay was performed (similar to that described by Rawlins *et al.*, 1985) with the above fusion proteins and a ^{32}P-labelled fragment encompassing the DNA binding region II. The latter was cloned from both B95-8 and C15 DNA using PCR and identities were checked by complete DNA sequence analyses. Sequence data showed only two nucleotide differences between C15 and B95-8 in this region of the viral genome with the changes occurring within the region of dyad symmetry (region II), as indicated (Figure 5). In the gel mobility retardation assay, the B95-8 (Figure 6, lanes 2 and 3) and C15 (Figure 6, lanes 5 and 6) fusion proteins bound strongly to their respective region II

DNAs, with no gross difference in binding affinity observed between the two. The specificity of the binding was confirmed using as controls either glutathione S transferase and region II DNA, or EBNA1 fusion protein from C15 and B95-8 with DNA from the open reading frame of EBNA1 itself (position 109410–109805) (Figure 6, lanes 7–11, respectively). In order to counteract non-specific interactions, the above reactions were repeated with 150 mM NaCl (final conc.) in the binding buffer; no differences in affinity were observed (data not shown). EBNA1 also formed complexes with region III DNA. As expected binding was much weaker than for region II (Jones *et al.*, 1989), but similarly no differences in binding affinity between C15 and B95-8 EBNA1 were observed (data not shown). There were no alterations in the region III DNA sequences from these two sources.

Discussion

The EBV nuclear antigen, EBNA1, plays a key role in the life cycle of the virus, as earlier studies on its DNA binding and episomal maintenance properties illustrated (Yates *et al.*, 1984, 1985; Lupton and Levine, 1985). Several laboratories have undertaken detailed analyses to define sites within the viral genome to which EBNA1 binds and the domain(s) of the protein responsible for binding. To date, three binding sites (strong affinity sites I and II, in *ori*P, and weak affinity site III, in *Bam*HI Q) have been identified (Jones *et al.*, 1989) and the functional activities for replication have all been mapped within the unique C-terminus of the protein, as summarised (Figure 3). This region also contains a potential basic–helix–loop–helix (B-HLH) structure associated with a number of DNA binding proteins (Inoue *et al.*, 1991), as well as a transactivation function and the EBNA1 nuclear localization signal (KRPRSPS, between amino acid residues 379–387; Ambinder *et al.*, 1991). Mutational analyses have identified a 16 amino acid core DNA recognition sequence (Chen *et al.*, 1994). Our data show that EBNA1 can also bind to RNA (Snudden *et al.*, 1994).

The multiplicity of key roles that can be attributed to this single viral function thus stimulated us to follow up our initial observation with RNase protection assays (Figure 1) which indicated heterogeneity within the EBNA1 gene from different sources. To investigate possible structural alterations that might lead to enzyme sensitivity in these assays, the sequences of the EBNA1 coding region were initially determined in the C15 xenograft, derived from a North African NPC (Busson *et al.*, 1988) and in a B-cell line (NAD-C15) generated with virus rescued from it (Fåhraeus *et al.*, 1988), then subsequently in numerous primary Asian NPC biopsies. (For practical reasons, this study did not include the highly repetitive domain between amino acids 90–358; transient replication and plasmid maintenance assays have shown that this region can be removed with little or no effect on functions of EBNA1) (Polvino-Bodnar *et al.*, 1988; Yates and Camiolo, 1988). The sequence results we obtained are summarised in Figure 2 and Table 1. They show numerous single base changes, many of which result in amino acid alterations relative to the same region in B95-8 cells. The mutations are not 'cell type' specific in that no differences are observed in the gene carried in the C15 NPC-related tumour and its B cell derivative, NAD. They may be indicative of strain variations; however, since only some of the base changes found in C15 are reflected in the Hong Kong NPCs (See Lung *et al.*, 1990; Abdel-Hamid *et al.*, 1992). With one exception (patient 58) sequences among Asian NPCs were essentially identical.

Most of the mutations that produced amino acid changes have been found to cluster in regions of the antigen containing N-terminal amino acids 16–27 and C-terminal residues 411–532 and 585–595 (numbering of Baer *et al.*, 1984). (Due to the complexity of the RNase protection assay patterns, Figure 1, it has proved difficult to interpret the bands seen with C15 and NAD-C15, however, with respect to these nucleotide changes.) Four of the mutations observed might affect the serine phosphorylation levels of EBNA1 (Hearing and Levine, 1985), three of them occurring in the C15 (glycine to serine, G to S, amino acid position 27; serine to cysteine, S to C, at 492 and alanine to serine, A to S, at 525) and one (threonine to serine, T to S, at 20) in the Hong Kong NPCs (see Figure 2 and Table 1); two of these (at positions 492 and 525) lie within the DNA binding domain of EBNA. A number of other mutations lie within a putative B-HLH DNA binding motif in EBNA1 (amino acids 467–597; Inoue *et al.*, 1991) or a corresponding AP-2 like helix-span-motif (HSH) (Sha *et al.*, 1992). Computer analyses (not shown) suggest

Table 1 The nucleotide changes observed in the EBNA 1 sequence of NPC and C15 relative to B95-8

Source	*Nucleotide No.*	*Nucleotide change*	*Amino acid change*
C15	107967	G > A	No change
NPC/C15	107998	G > C	E > Q
NPC/C15	108005	G > A	G > E
NPC	108010	A > T	T > S
C15	108024	A > C	E > D
C15	108031	G > A	G > S
NPC/C15	108060	T > G	No change
NPC/C15	108120	C > T	No change
NPC/C15	109083	A > T	No change
C15	109183	G > C	E > Q
NPC	109185	A > T	E > D
NPC	109205	A > T	H > L
NPC (No. 58)	109233	T > C	No change
C15	109237	G > A	V > M
NPC	109267	G > A	A > T
C15	109379	C > A	P > Q
C15	109401	A > C	E > D
C15	109411	G > A	A > T
NPC	109412	C > T	A > V
C15	109426	A > T	S > C
C15	109449	C > T	No change
NPC	109449	C > G	D > E
NPC	109457	C > A	T > N
NPC/C15	109512	A > C	No change
NPC/C15	109523	C > T	T > I
C15	109525	G > T	A > S
NPC	109534	A > G	I > V
NPC	109549	C > A	L > I
NPC/C15	109611	G > A	No change
C15	109705	A > C	T > P
NPC (No. 58)	109706	C > T	T > I
NPC/C15	109733	G > A	R > K
C15	109736	T > C	V > A
C15	109816-109824	Duplicated	DDGDDG

that the changes observed are, however, allowed for by degeneracies in B-HLH sequences (Inoue *et al.*, 1991). An insertion at the extreme C-terminus, where duplication of a DDG sequence already exists, resulted in three copies of this DGG triplet sequence in the C15 genome. The latter region has been identified as having homology with an acidic nuclear intermediate early gene (IEI) of cytomegalovirus, involved in association with metaphase chromosomes (Lafemina *et al.*, 1989). No mutations were observed in the region associated with RNA binding (Snudden *et al.*, 1994).

Since many mutations were clustered in regions associated with DNA binding (see Figure 3), we performed gel retardation assays as previously described by Rawlins *et al.* (1985), examining the ability of the C-termini of B95-8 and C15 EBNA1 to bind DNA from their respective genomes and to retard its mobility on gels. For this study, binding to the region of dyad symmetry (region II DNA) was chosen since it contains the site for the initiation of viral DNA replication in latently infected cells and is essentially independent of binding to region I (or presumably III) (Jones *et al.*, 1989; Hearing *et al.*, 1992). We postulated that such binding might be very sensitive to sequence alterations and we determined the sequence of the dyad symmetry region in C15 since it could carry mutations relevant to binding by C15 EBNA1. Two point mutations were indeed identified, one in site 3 that might possibly destabilize a potential hairpin structure, and one in site 2 which would be predicted to have little effect (see Figure 5). The C-termini of both B95-8 and C15 (expressed as fusion proteins with glutathione S-transferase using the GEX expression system, and purified to near homogeneity) were used in mobility retardation assays. Our data showed that no significant differences were observed in affinity binding. That is, within any particular cell type, the mutations (Figures 2 and 5) were not reflected in the DNA binding studies, each EBNA1 binding to its corresponding DNA site, with no gross affinity differences being observed (Figure 6). Likewise, in binding studies with Region III (data not shown), we saw no differences in binding between B95-8 and C15 EBNA1's (although as this is a weakly binding region, small variations might not have been detected). Our data are thus consistent with a recently described core DNA recognition sequence (Chen *et al.*, 1994) in that none of the naturally-occurring mutations we observe lie within this core structure (amino acids 459–475). The effect of mutations in the N-terminus of EBNA1 (amino acids 16–27) were not investigated in this assay since Yates and Camiolo (1988) showed that a deletion over this region (mutant DL98) had only marginal effects on the activity of EBNA1 in either plasmid maintenance or DNA replication, or on its ability to act as a transcriptional enhancer. One value of our experiments is that they support the assumption that studies on DNA replication and plasmid maintenance in a model system (B95-8) may with more confidence be extrapolated to human cells.

A simplistic explanation for the mutations observed in the EBNA1 open reading frame from human, as opposed to marmoset (B95-8), cells is that they have no effect upon function. However, this seems unlikely for a protein as complex and important to the biology of EBV as EBNA1. To date, in seeking for the functional significance for observed sequence changes in EB in the tumours, we have analysed DNA binding as it relates to viral replication. Another area needs exploration is that involving transcripti control. Whereas in B95-8 cells, viral gene expres is relatively promiscuous, some cells undergoing full lytic cycle, in EBV-associated tumour cells, v transcription has been shown to be tightly control with all the known EBV nuclear functions, s EBNA1, being silent (Hitt *et al.*, 1989; Gilligan *et a* 1990; Chen *et al.*, 1992; Zhang *et al.*, 1993). This is line with data on protein expression in Burkit lymphoma and NPCs (Rowe *et al.*, 1986; Fåhraeus *al.*, 1988). (Even in the lymphoblastoid line, NA there is evidence for controlled transcriptional expr sion and no virus appears to be generated up induction; Hitt *et al.*, 1989 and our unpublish data.) In addition to regulating its own expressio (Sample *et al.*, 1992), EBNA1 may act to dow regulate nuclear or other functions, either at t transcriptional or post-transcriptional level. Several o the consistently observed naturally-occurring s mutations (at amino acid numbers 487, 524, 585 an 594; Figure 2) lie within a broadly mapped regio associated with a transactivator function (Figure 3 Thus, transactivation remains an EBNA1 function th might be affected by the observed changes. On th other hand, two consistently observed alterations at th N-terminus of the antigen, at amino acids 16 and 1 lie in regions where no activities have been assigne Thus, we cannot exclude the possibility that severa other key functions associated with the antigen (fo example, relating to oncogenesis or cellular immun escape), or even activities yet to be identified, ar reflected in the observed mutations. Experiments t distinguish among the various possibilities raised mus form the basis of future work.

Materials and methods

Cells

The C15 tumour is a human nasopharyngeal carcinoma of North African origin (Busson *et al.*, 1988), maintained by propagation in nude mice. B95-8 is an EBV-infected marmoset cell line, permissive for viral replication. NAD-C15-STO-B (referred to subsequently as NAD-C15) is a B lymphoblastoid cell line derived from co-cultivation of normal adult cells with the C15 tumour (Fåhraeus *et al.*, 1988). All cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine. NPC biopsy materials from different patients presenting with tumour, at various times, were kindly provided by Drs D Choy and J Sham, Queen Mary Hospital, Hong Kong; DNA was isolated from biopsies in Hong Kong and sequence analyses performed in London.

DNA and RNA extractions

DNA and RNA was isolated as previously described by Hitt *et al.* (1989a).

Ribonuclease protection assay

The method used was essentially that described by Sambrook *et al.* (1989). Briefly, a single-stranded ^{32}P-radiolabelled RNA probe containing sequences between the SacI/SacII sites in the C terminal region of EBNA1

positions 109410–109804 in the B95-8 EBV sequence (Baer *et al.*, 1984)] was hybridised overnight at 45°C to 1 μg of RNA from B95-8, C15 and NAD-C15. The hybrid was then digested at 37°C with 300 μl of RNase digestion mixture containing 300 mM NaCl, 10 mM Tris.HCl (pH 7.4), 5 mM EDTA, 2 μg of RNase T1 per ml and 40 μg of RNase A per ml. The samples were then treated with proteinase K, extracted with phenol-chloroform and precipitated with ethanol. The pellet was resuspended in sample buffer, separated on a 7.5% polyacrylamide sequencing gel, dried and subjected to autoradiography.

The polymerase chain reaction

The Polymerase Chain Reaction (PCR) was carried out using 200 ng of template DNA and *Taq* polymerase (Perkin Elmer Cetus), as recommended by the manufacturer. The reaction mixtures were incubated at 94°C, 1 min; 50°C, 1 min; 72°C, 2 min. After 30 cycles, the polymerization step at 72°C was extended to several minutes to complete all strands. The reaction was analysed by running a small aliquot on a 0.8% mini-gel. The amplified DNA was then eluted from the gel, digested with the appropriate enzymes and ligated into a suitable vector.

Amplification of DNA encoding EBNA1

DNA from C15, NAD-C15, B95-8 and NPC biopsies was used for amplification by PCR of the EBNA1 open reading frame (ORF). A total of 7 NPC biopsies were studied (numbered 58 to 64). The location of the oligonucleotide pairs used in the PCR with respect to the EBNA-1-ORF is shown in Figure 2. The primers include up to 11bp extensions containing a restriction site (*Bam*HI or *Eco*RI, GGATCC and GAATTC, respectively) to facilitate cloning of the PCR product. Their sequence and position in the B95-8 EBV genome (Baer *et al.*, 1984) is given below:

5'-GTA*GGATCC*CCATGTCTGACGAG (oligonucleotide 1; position 107950)
5'-GTC*GAATTC*CACCGTGGGTC (oligo 2; position 108213)

5'-AC*GGATCC*CGTGAAAGAGCCAGGGGGGGA (oligo 3; position 109021)
5'-CGC*GAATTC*GGTAGTCCTTTCTACGT (oligo 4; position 109445)

5'-TA*GGATCC*AGAACATTGCAGAAGGT (oligo 5; position 109385)
5'-CGG*GAATTC*ACGGCTTTTAATAC (oligo 6; position 109925)

In the case of oligonucleotide 6, a single base change (A to T at position 109919) generated an *Eco*RI site.

The amplified DNA was eluted from a 0.8% agarose gel, digested with the appropriate enzymes and cloned into M13 vectors for sequencing according to standard protocols. The unique N- and C-terminal sequences from C15 and seven primary Hong Kong NPC biopsies were determined from PCR-derived clones, and compared with the sequence obtained from B95-8 marmoset cells using the same protocol. The sequences of several clones per sample were determined, to allow for potential PCR artefacts. Sequence analyses were performed over the course of a year, as materials became available.

Synthesis and purification of EBNA1 fusion protein

PCR was used to amplify the C terminal EBNA1 coding region from both B958 and C15 DNAs. The oligonucleotides used were: 5' - TCATCCGGATCCCCACCGCGCAGG (position 109117) and 5' - CGGGAATTCACGGCTTTTAATAC (position 109925). For cloning purposes the first oligonucleotide primer includes a G > A and T > C base change at positions 109125 and 109128 which generates a *Bam*HI restriction site and the second oligonucleotide includes an A > T base change at position 109910 which gives an *Eco*RI site. After verification by sequencing, the fragment was inserted in-frame behind the C terminus of Sj 26, a 27.5 kD glutathione S-transferase in the GEX expression plasmid. Screening of transformants and large-scale purification of the EBNA1 fusion protein was carried out as described by Smith and Johnson (1988). The protein was resolved by SDS–PAGE (12% polyacrylamide gel) as described by Laemmli *et al.* (1970) and then electrotransferred onto a nitrocellulose membrane filter in blot buffer (25 mM Tris, 192 mM glycine (pH 8.3), 20% v/v methanol) in a cooled tank overnight (8 V/cm). The filter was washed in PBS containing 0.05% Tween (PT) then incubated for 30 min with PBS containing 0.05% Tween 20 and 5% fat-free milk powder (PTM) with gentle rocking. Protein blots were incubated with a 1:50 dilution (in PTM) or rabbit polyclonal antibody against EBNA1 (Hearing *et al.*, 1985) for 90 min at room temperature, washed in PT three times for 20 min and then reacted with 1 in 500 dilution of rabbit anti-mouse immunoglobulin (Ig)-peroxidase conjugate (DAKO PATTS). After being washed in PT as described above the filter was developed with 3,3'-diaminobenzidine tetrahydrochloride (200 μl DAB (20 mg ml^{-1}), 9.7 ml PBS, 100 μl 1% $CoCl_2$, 1%$Ni(NH_4)_2$ $(SO_4)_2$, 10 μl H_2O_2 (30% w/v)).

^{32}P incorporation into DNA binding substrate

PCR was used to clone two known EBNA1 DNA binding regions, the region of dyad symmetry (region II) localised to *Bam*HI C and the lower affinity binding site region III within *Bam*HI Q (Rawlins *et al.*, 1985). Amplification of regions II and III from B95-8 and C15 DNA was performed using the following oligonucleotide primers:

Region II oligonucleotides

5'-CCAT*GAATTC*GTGTGAGATG (position 8572)
5'-ATAA*GGATCC*CCTTGTTAAC (position 9150)

Region III oligonucleotides

5'-CTATAACGCAGGTCCTGTTC (position 62202)
5'-CAAT*GAATTC*CGGCTCGGAG (position 62616)

Nucleotides typed in bold have replaced B95-8 sequence to generate restriction sites (*Eco*RI, GAATTC, or *Bam*HI, GGATCC) for use in cloning. In the case of the first region III oligonucleotide, creation of a restriction site was not necessary as the *Bam*HI site in the EBV sequence 50 bases downstream of the primer was used for cloning purposes. After binding regions were verified by sequence analysis, they were subcloned into the Bluescribe vector (Stratagene). Plasmid DNA was then cleaved with *Bam*HI and *Eco*RI restriction enzymes and 'end-filled' with [^{32}P]dCTP using the Klenow fragment of DNA polymerase. The labelled fragments representing the DNA binding regions were purified by elution from a 0.8% agarose gel followed by phenol-chloroform extraction and ethanol precipitation. End-labelled DNA was then used in a mobility retardation assay.

Mobility retardation assay for DNA binding

Protein-DNA complexes were formed by mixing 0–5.2 μg fusion protein with 1×10^4 c.p.m. of ^{32}P-labelled probe in

20 μl binding buffer (25 mM Tris.HCl (pH 8.0), 50 mM NaCl, 1 mM $MgCl_2$, 5 mM spermidine, 0.5 mM dithiothreitol, 5% v/v glycerol. After incubation at 30°C for 20 min the samples were loaded on a 4% non-denaturing polyacrylamide gel (acrylamide:bisacrylamide ratio of 19:1) in 50 mM Tris-glycine, pH 8.8. Electrophoresis was performed at 180V for ~2 h at room temperature then gels were dried and exposed to X-ray film at −80°C using intensifying screens.

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AB Vascular endothelial cells are suspected of being the target of autoimmune processes seen in many connective tissue diseases and in systemic vasculitis as evidenced by the detection of circulating autoantibodies against endothelial cell antigens. To select B cells recognizing endothelial cells antigens, Epstein-Barr virus (EBV)-infected B

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CELLULAR IMMUNOLOGY **150**, 15–26 (1993)

Production of Anti-endothelial Cell Antibodies by Coculture of EBV-Infected Human B Cells with Endothelial Cells[1]

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Vascular endothelial cells are suspected of being the target of autoimmune processes seen in many connective tissue diseases and in systemic vasculitis as evidenced by the detection of circulating autoantibodies against endothelial cell antigens. In order to select B cells recognizing endothelial cells antigens, Epstein–Barr virus (EBV)-infected B cells, obtained from one patient presenting a systemic vasculitis, were cocultured with human endothelial cells concurrently with a human endothelial cell line (EC-pSV1 cells). This coculture consisted of a first step of expansion of B cells specifically selected by adherence onto human umbilical vein endothelial cells (HUVEC). The adherence of selected B cells was specific to endothelial cells because no rosette formation around control cells (HeLa cells or COS cells) was observed. Adherent B cells were cloned by limiting dilution by coculture onto EC-pSV1 cells and screened for anti-HUVEC antibody production by endothelial cell ELISA. An increase in anti-HUVEC antibody production of IgM isotype was detected by endothelial cell ELISA, peaking at Day 9 and remaining constantly elevated, relative to B cell expansion. Among 21 B cell lines producing IgM, 6 presented high levels of anti-HUVEC antibodies, whereas 1 of 52 B cells cloned without EC-pSV1 cells showed such antibody production. Anti-HUVEC antibody production and B cell proliferation were dependent on the presence of endothelial cells. Two of these 6 B cell lines produced antibodies directed against an endothelial cell antigen with an apparent molecular weight of 192 kDa as determined by immunoblotting analysis. Our results demonstrate that adherence of EBV-infected B cells to endothelial cells and further cloning by adherence can efficiently select anti-HUVEC antibody-producing human B cells and might help to define antigens potentially involved in autoimmune diseases.

INTRODUCTION

Vascular damage is a common feature in many connective tissue diseases and in systemic vasculitis. The presence of circulating autoantibodies directed against endothelial cell components has led to attractive hypotheses involving these antibodies in the pathogenesis of many diseases such as systemic lupus erythematosus (1–3), rheumatoid arthritis (3, 4), progressive systemic sclerosis (3, 5), autoimmune hypoparathyroidism (6), episodic angioedema and eosinophilia syndrome (7), systemic

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vasculitis (8), allergic and granulomatosus angiitis (9), and Kawasaki disease (10, 11). However, a close relationship between the presence of such antibodies and the development of the underlying disease remains unclear, which justifies a more precise evaluation of recognized endothelial cell epitopes.

Attempts to characterize the endothelial cell target antigens have met with some difficulties for two reasons: (i) the limited quantity of cultured human endothelial cells available for biochemical purification and (ii) the large diversity of serum antibodies. Due to these technical problems, the characterization of endothelial cell antigens remains a difficult issue.

The ability of human B cells to proliferate and to produce antibodies following infection with Epstein–Barr virus (EBV) has been widely used to study human antibodies putatively involved in autoimmune diseases. In this way, human monoclonal antibodies have been raised against DNA (12, 13), immunoglobulin (14), tetanus toxoid (15, 16), thyroglobulin (16), blood group antigens (17), or acetylcholine esterase (18). However, the relative paucity of circulating B cells with a given specificity strongly reduces the probability of obtaining B cell lines secreting antibodies of interest.

One alternative approach to selecting specific B cells is to take advantage of the fact that B lymphocytes express immunoglobulins on their surface. Thus, specific B cells have been successfully separated from irrelevant B cells using the antigen as a probe (16).

In this report, we used the method of selection by cellular adherence to identify antigenic determinants from endothelial cells with human B cell lines. EBV-infected B cells were selected by adherence to human endothelial cells and then cocultured. Coculture of adherent B cells in limiting dilution led to the generation of a number of antibodies against endothelial cells. Two of them were able to recognize an endothelial cell antigen of 192 kDa.

MATERIAL AND METHODS

Immortalization of B Lymphocytes by EBV Infection

B cells were provided by a patient suffering from allergic granulomatosus and angiitis, also called Churg and Strauss syndrome (19). This syndrome is characterized by severe asthma, diffuse symptoms of systemic vasculitis, and blood and tissue eosinophilia. The clinical diagnosis was confirmed in this patient by neuromuscular biopsy, which demonstrated necrotic, perivascular, and extravascular granulomas rich in eosinophils.

Mononuclear leukocytes were prepared from 60 ml heparinized peripheral blood by density centrifugation on Ficoll–Hypaque (Pharmacia Biochemicals, Inc., Upsala, Sweden) (20). Following monocyte depletion by 2 hr of adherence to plastic dishes (Becton–Dickinson, Grenoble, France), T lymphocytes were depleted by density sedimentation of rosette formation with sheep red blood cells treated with 2-aminoethylisothiouronium bromide. The B lymphocytes were cultured in 24-well cell culture plates to a concentration of 1×10^5 cells/well in 1 ml of RPMI medium containing 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL, Cergy Pontoise, France), 2 m*M* L-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. To each well, 100 μl of supernatant from a dense culture of an EBV-infected marmoset cell line (B 95-8) (ATCC, Rockville, MD) was added (21, 22). B lymphocytes were cultured 2 weeks until colony formation was observed and were expanded one more week before selection by coculture.

Cell Cultures

Human endothelial cells were derived from umbilical vein (HUVEC), according to the method previously described (23, 24). Briefly, HUVEC were collected after treatment of umbilical vein by 0.2% collagenase in Hanks' balanced salt solution for 15 min (M. A. Bioproducts, Walkerville, MD) and pelleted by centrifugation (800*g*, 10 min). HUVEC were resuspended at 1.2×10^5 cells/ml in RPMI 1640 supplemented with 2 m*M* L-glutamin, 100 U/ml penicillin, 10 μg/ml streptomycin, 20% FCS (v/v), 100 μg/ml heparin, and 25 μg/ml endothelial cell growth supplement (Sigma Chemical Co., St. Louis, MO). HUVEC were cultured in 35-mm-diameter tissue culture wells at 37°C in 5% CO_2. The culture reached confluency within 3 to 5 days. Only endothelial cell cultures obtained after the second to the fourth passage were used.

Simian virus 40-transfected endothelial cell line (EC-pSV1 cells) (25) was cultured, as well as HeLa cells, in RPMI 1640 supplemented with 2 m*M* L-glutamine and 10% (v/v) FCS. COS cells and MRC5 cells were cultured in Dulbecco's medium supplemented with 2 m*M* L-glutamine and 10% (v/v) FCS.

Coculture and Cloning Procedure

In order to select B cells able to recognize endothelial cell antigens, EBV-infected B cells were cocultured with EC-pSV1 cells, which were used because they are easy to obtain in large amounts in comparison with classical HUVEC culture. In the first step, EBV-infected B cells (2×10^7 cells) were added to three 100-mm-diameter petri dishes containing confluent EC-pSV1 cells (in EC-pSV1 cell culture medium) and then incubated at 37°C in 5% CO_2 for 14 days. Medium containing the nonadherent B cells was removed by three washings every 2 to 3 days. Following centrifugation, supernatants were aliquoted and stored at −20°C until tested for the presence of immunoglobulins.

In a second step, adherent B cells recovered after vigorous washings were seeded twice a week, in order to expand the EBV-infected B cells. The resuspension was controlled by optical microscopy examination of the petri dishes, which confirmed the dramatic decrease in the number of B cell–EC-pSV1 cell rosettes. An aliquot of the adherent B cells was used for limiting dilution assay at 0.5 cell/well in four 96-well flat-bottom microculture plates containing confluent EC-pSV1 cells. The medium was changed twice a week. Detection of anti-HUVEC antibodies in culture supernatants was performed each week during three consecutive weeks of coculture. B cells in wells containing anti-HUVEC antibodies, or derived from wells not containing such antibodies but presenting a strong proliferative response, were expanded in 16- and then 36-mm-diameter culture plates. B cells cultured and further cloned by limiting dilution without coculture with EC-pSV1 cells as described above were used as controls.

Quantification of Immunoglobulin Production by ELISA

Microtiter plates were coated with anti-human heavy-chain goat antibodies specific for human IgG, IgA, and IgM (Diagnostics Pasteur, Marnes la Coquette, France). One hundred microliters of coculture supernatants was incubated for 1 hr at room temperature in duplicate. After extensive washes in PBS containing 0.05% (v/v) Tween 20, wells were then incubated for 1 hr with affinity-purified and peroxidase-conjugated anti-human heavy-chain goat antibodies (Tago, Burlingame, CA). Following extensive

washings, the wells were allowed to react with 2 mg/ml of the substrate *o*-phenylenediamine in 10 m*M* citrate buffer, pH 4.2, containing 0.075% (v/v) H_2O_2. The reaction was stopped by adding 100 μl 4 *N* HCl per well. The standards were made of various dilutions of purified human IgA, IgG, and IgM (Sigma Chemical Co.). The optical density was read at 492 nm and the results were expressed in nanograms per milliliter.

Adherence Assay

A total of 0.1×10^6 cells (HUVEC, EC-pSV1 cells, COS cells, and HeLa cells) were seeded on gelatin-coated 8-well tissue culture chamber slides in duplicate for 24 hr (Nunc Inc., Naperville, IL). The slides were washed by dipping them in a glass flask containing 100 ml RPMI 1640 prewarmed to 37°C. Then, 10^5 EBV-transformed B cells were added immediately to each well. Following 5 min of incubation at 37°C, the slides were washed again three times in order to remove the nonadherent B cells and immediately fixed in PBS/glutaraldehyde [0.5% (v/v)] for 30 min at 4°C. After extensive washes, the slides were examined under microscope to quantify the number of rosettes. A rosette was taken in account when more than five B cells were fixed around target cells (endothelial cells or control cell lines COS and HeLa). In order to identify the mechanism of the adherence pathway, the same adherence experiments were repeated with a previous 2-hr incubation of endothelial cells with 2 μg/well monoclonal anti-ICAM-1 or anti-ELAM-1 antibodies (British Biotechnology Ltd, Oxford, UK).

Detection of Anti-HUVEC Antibodies by ELISA

Confluent cultures of HUVEC on gelatin-coated 96-well flat-bottom microculture plates were washed twice with PBS and then fixed with 0.05% glutaraldehyde in PBS for 10 min at 4°C (26). Following four washings in PBS containing 1% BSA and 5 m*M* EDTA, the plates were incubated at room temperature for 1 hr in the same buffer. After washings, 100 μl of coculture supernatants was incubated for 1 hr at room temperature and then washed four times before incubation with affinity-purified and peroxidase-conjugated anti-human IgA, IgM, or IgG goat antibodies. Following extensive washings, the wells were allowed to react with 2 mg/ml of the substrate *o*-phenylenediamine in 10 m*M* citrate buffer, pH 4.2, containing 0.075% H_2O_2. The reaction was stopped by adding 100 μl 4 *N* HCl per well. The optical density values were read at 492 nm and the results were expressed as follows: OD (sample) − mean value of all OD (0.135 ± 0.107; mean of 288 values subtracted from OD values up to 1.0). Purified human IgG, IgA, and IgM were used as standards. In our system, purified immunoglobulins at concentration levels fivefold greater than those found in coculture supernatants did not efficiently bind to HUVEC.

Control was established by anti-MRC5 cell ELISA; this ELISA was used under the same conditions as those described for endothelial cells.

Analysis of Antigens Recognized by Anti-HUVEC Antibodies

Antigenic extracts from EC-pSV1 cells, HUVEC, monocytes, lymphocytes, neutrophils, HeLa cells, COS cells, and MRC5 cells were prepared. After cell collection, the cells were centrifuged and resuspended at a concentration of 2×10^8 cells/ml in ice-cold lysis buffer (0.1 *M* Tris–HCl, pH 7.4, 150 m*M* NaCl, 10 m*M* $MgCl_2$) containing

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0.5% (v/v) Nonidet-P40. The extracts devoided of nucleus fraction were fractionated on a 13% polyacrylamide slab gel containing 0.1% SDS under reducing conditions as described by Laemmli (27). After transfer of separated proteins onto nitrocellulose (0.45 μm BA85, Schleicher & Schuell, Dassel, Germany) (28), sheets were saturated in quenching buffer (5% nonfat dry milk in PBS, pH 7.4) for 30 min at room temperature and after several washings incubated with coculture supernatants diluted at 1:2 or with the patient serum diluted at 1:100 in PBS for 18 hr at 4°C under constant agitation. After washings, bound human immunoglobulins were detected with peroxidase-conjugated anti-human immunoglobulin goat antibodies. After a final washing step, nitrocellulose membrane was stained with 0.06% (v/v) 4-chloro-1-naphthol/0.075% (v/v) H_2O_2 in PBS, pH 7.4 (Bio-Rad Laboratories, Richmond, CA). The anti-192-kDa antibodies were tested for their putative blood group antigen recognition. The search for anti-A and anti-B blood group antibodies and abnormal blood group antigens was realized by Coomb's method and the papain method using standardized panels of red blood cells obtained from the Centre Regional de Transfusion Sanguine (CRTS de Lille, Lille, France).

RESULTS

Proliferation of Adherent B Cells Was Associated with the Production of Anti-HUVEC Antibody

During the first 2 weeks of selection, the evolution of the IgM anti-HUVEC antibody response of B cells adherent to EC-pSV1 cells was analyzed (Fig. 1). At the beginning of the coculture, we observed a critical decline in anti-HUVEC antibody activity at Day 4. The optical microscopic examination at that time revealed that up to 90% of B cells were removed by washings. Only few rosettes of B cells around EC-pSV1 cells could be seen. At Day 6, anti-HUVEC antibodies increased and reached a plateau at Day 9. In contrast, anti-HUVEC antibodies found in supernatants of EBV-transformed B cells cultured alone were lower than those found in coculture supernatants, without any change during the time of culture. In this case, no band could be evidenced by

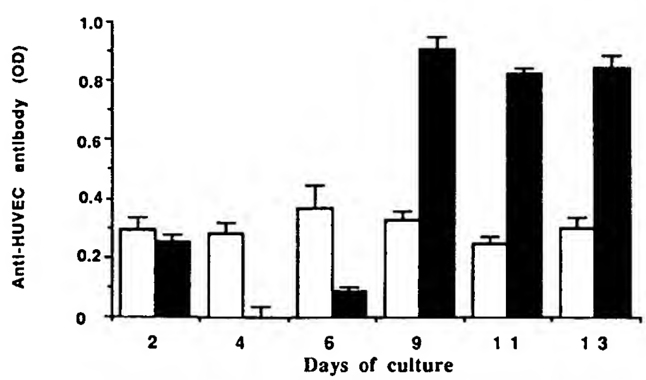

FIG. 1. Time course of the appearance of human IgM anti-endothelial cell antibodies in the supernatants of EBV-infected B cells cocultured with EC-pSV1 (■). Controls supernatants were made of supernatants from EBV-infected B cells cultured alone (□). The results are given in optical density values (OD) as means ± SD of three experiments.

immunoblotting analysis against HUVEC extracts. Immunoblot analysis of the coculture cell supernatants collected at Day 9 against endothelial cell extracts revealed the presence of 23 bands ranging from 17 to 200 kDa (data not shown). This increase of anti-HUVEC antibody level at Day 9 corresponded to a strong rise in B cell rosette formation around EC-pSV1 cells. This observation was confirmed by an adherence assay of EBV-transformed B cells collected at Day 9 to EC-pSV1 cells, HUVEC, COS cells, or HeLa cells. The results demonstrated that 91 ± 5% EC-pSV1 cells and HUVEC formed rosettes, while only 4 ± 2% HeLa cells and COS cells did (Fig. 2). Moreover, addition of anti-ICAM-1 antibody dramatically decreased the adhesion of B cells onto EC-pSV1 or HUVEC (5 ± 2%), whereas no variation was observed on addition of anti-ELAM-1 antibody (data not shown).

Production of Immunoglobulins and Anti-HUVEC Antibodies by Adherent B Cells

Among a total of 288 wells tested, only 28 produced detectable anti-HUVEC antibodies. During clonal expansion, 7 clones ceased to proliferate (Fig. 3A). Among these 21 remaining lines, the majority produced immunoglobulins of IgM isotype, except for 4 B cells that produced antibodies of IgA and IgG classes (Fig. 3B). Twenty-one clones were maintained for up to 3 months, and 4 of them showed strong proliferative responses for up to 8 months (3G9, 3G11, 3G12, 4D4). Numerous supernatants of B cell clones demonstrated a low binding activity to MRC5 cells in contrast to the binding activity observed with HUVEC (Fig. 3A). Moreover, nonadherent B cells, also cloned by the limiting dilution technique without EC-pSV1 cells, failed to produce

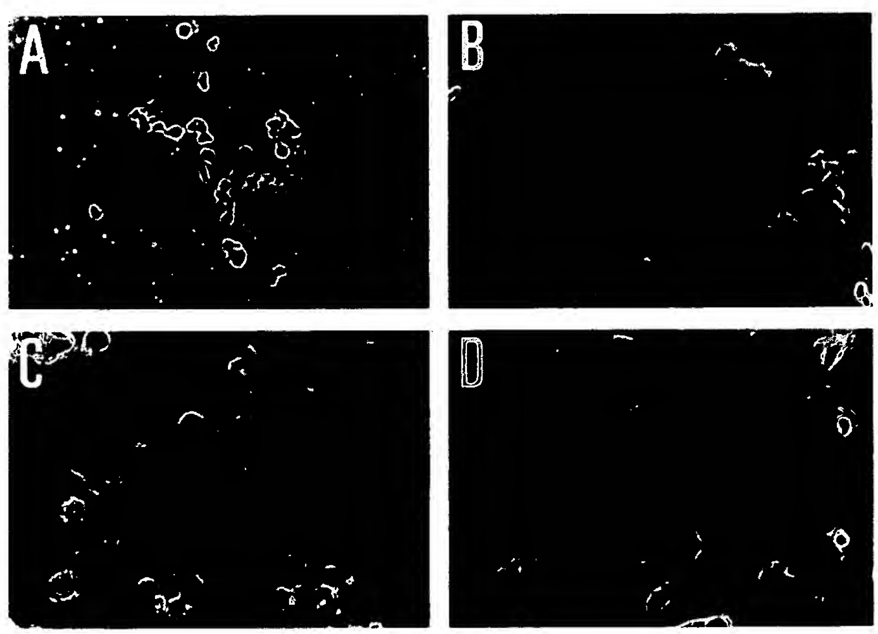

FIG. 2. Binding of the EBV-infected B cells on HUVEC (A), EC-pSV1 cells (B), HeLa cells (C), and COS cells (D). Five minutes of incubation of adherent B cells allowed the formation of typical rosettes with 91 ± 5% HUVEC or EC-pSV1 cells. COS cells and HeLa cells did not support such adhesion properties (4 ± 2%).

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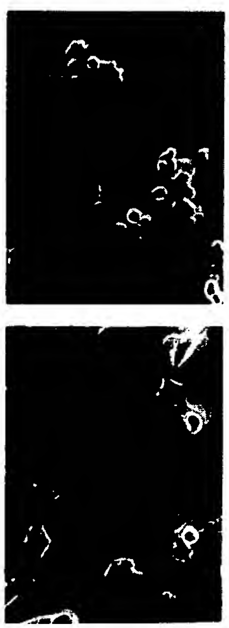

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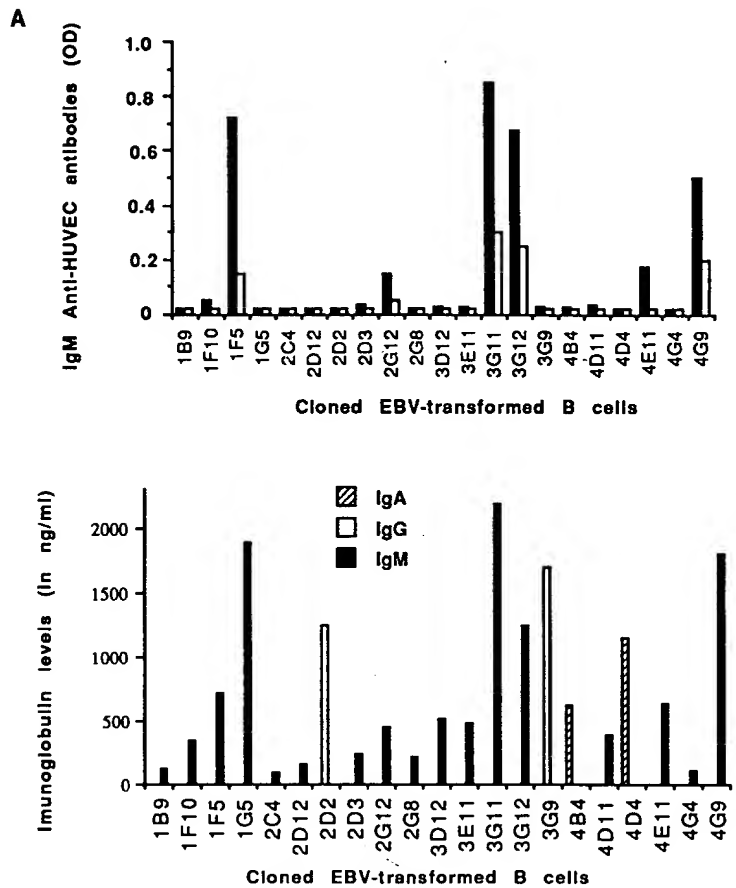

FIG. 3. Production of antibodies by the selected EBV-transformed B cells cloned by the limiting dilution technique. (A) The human IgM antibodies produced by the 21 B cell lines were directed against HUVEC (■) but not to MRC5 cells (□) as detected by ELISA, in which results are expressed as the mean OD from a duplicate after subtraction of the mean value of all OD (0.135 ± 0.107). (B) Isotype analysis of immunoglobulins produced after 72 hr of culture, in which results are expressed in ng/ml of immunoglobulin and are representative of three measurements.

anti-endothelial cell antibodies: after 21 days of culture, 52 proliferative lines among 576 wells were obtained. Although the majority of these cell lines produced immunoglobulin of μ class, only 1 of the 52 cell lines showed a weak anti-HUVEC antibody level (OD values range: 0.1–0.2) (data not shown).

Endothelial Cell-Dependent Growth and Immunoglobulin Production by Cloned EBV-Infected B Cells

When B cells cloned by coculture with EC-pSV1 cells were cultured alone, the anti-HUVEC antibody production decreased quickly, and the B cells no longer proliferated as well as they did under coculture conditions (Fig. 4A). The inability of cloned B cells to proliferate without the presence of endothelial cells led us to investigate a putative role for EC-pSV1-derived mediators, such as interleukin 6 (IL-6), which is

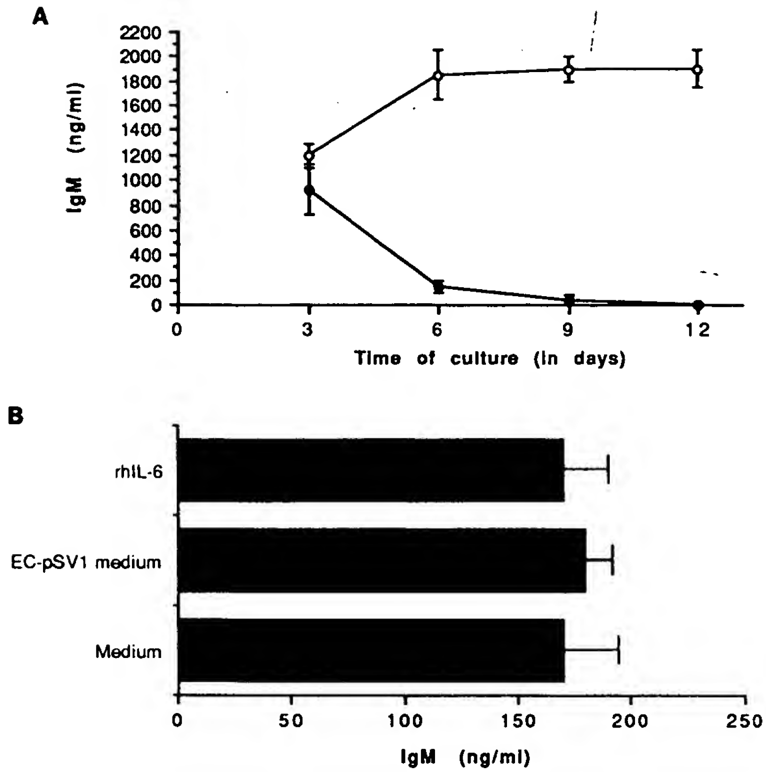

FIG. 4. Role of EC-pSV1 in the proliferation of B cells. (A) Time course of IgM production by the B cell line 3G11 cultured with EC-pSV1 (○) or alone (●); supernatants were collected at 72 hr and results are expressed in ng/ml. Results are similar for the other B cell lines tested. (B) Inability of rhIL-6 or EC-pSV1 conditioned medium to replace EC-pSV1 in IgM production by the cloned B cell lines. Results are expressed in ng/ml.

known to be an autocrine B cell growth factor produced by EBV-infected B cells and to be produced in large quantities by EC-pSV1 cells (25). The proliferation and the immunoglobulin production of the 3G11 cloned B cell line, when cultured without EC-pSV1 cells, quickly decreased at Day 3. Addition of recombinant human IL-6 (5000 U/ml), or EC-pSV1-conditioned medium (collected from a 72-hr culture of EC-pSV1 cells), was unable to stimulate proliferation and immunoglobulin production of cloned B cells regardless of the time of culture (Fig. 4B). At Day 12, no viable B cells could be detected. Similar results have been obtained with 4 other B cell lines (1G5, 3G9, 3G12, and 4D4) producing IgM antibodies against HUVEC.

Detection of Human Anti-192-kDa Endothelial Cell Antigen Antibody

The presence of a high antibody level in supernatants of some cloned B cells has led to the identification of the endothelial cell target antigens recognized by these antibodies. The immunoblot analysis of supernatants of B cell lines against HUVEC extracts revealed the presence of an endothelial cell antigen with an apparent molecular weight of 192 kDa, as observed with two B cell lines (3G11 and 3G12) (Fig. 5). A

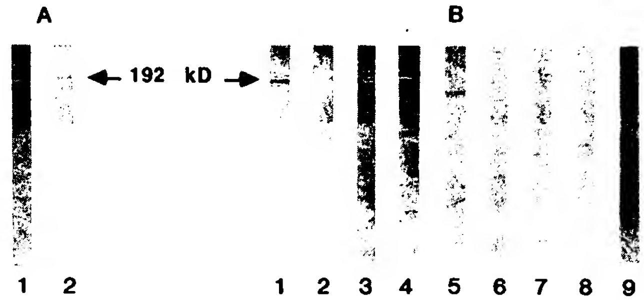

FIG. 5. Detection of an endothelial cell antigen as a target for antibodies of isotype produced by the B cell lines. (A) Immunoblotting analysis against HUVEC extracts revealed that a 192-kDa endothelial cell antigen was recognized by the anti-HUVEC antibodies of IgM isotype produced by the B cell lines 3G11 (1) and 3G12 (2). (B) A 192-kDa antigen was detected by the anti-HUVEC antibodies contained in the coculture supernatant of the B cell line 3G11 in EC-pSV1 (1), in HUVEC (2), and in nonadherent PBMC antigenic extracts (3). A 200-kDa antigen and a 160-kDa antigen were detected by these supernatants in mononuclear cell extracts (4) and platelet extracts (5), respectively. No other antigens were evidenced in cellular extracts including COS cells (6), MRC5 cells (7), HeLa cells (8), or PMN cells (9).

band of the same molecular weight was detected when the immunoblots were performed with cell samples prepared under reducing and unreducing conditions (data not shown). This 192-kDa antigen was also detected in EC-pSV1 cells. The anti-HUVEC antibody present in supernatant 3G11 was demonstrated to recognize an adherent PBMC antigen with an apparent molecular weight of 200–210 kDa, a platelet antigen of 160–170 kDa, and an antigen with the same molecular weight of 192 kDa present in lymphocytes (Fig. 5). Further separation of T and B lymphocytes by rosette formation followed by T and B cell sampling under reducing conditions showed that the 192-kDa antigen was present on both resting T and B cells (data not shown). This antigen was not detected in neutrophils or HeLa cell, COS cell, or MRC5 cell extracts and no blood group antigen was recognized by the anti-192-kDa antibody (data not shown).

DISCUSSION

Until now, to our knowledge, no human B cell epitope from endothelial cells recognized by autoantibodies has been identified. Our goal was to favor the production of such antibodies used as probes to identify the endothelial cell antigens that might putatively be involved in human autoimmune diseases. In the present study, we used a method of producing antibodies to human endothelial cells from a human B cell repertoire, selected by adherence to endothelial cells. Two of the B cell lines produced antibodies of IgM isotype which recognized an endothelial cell antigen with an apparent molecular weight of 192 kDa.

Whether the anti-HUVEC antibody-producing B cells selected in our system reflect the anti-HUVEC serum antibodies detected in different autoimmune diseases remains unresolved. The B cell population used in this study was obtained from a patient who suffered from Churg and Strauss syndrome (19). We speculated that in this syndrome, as in systemic vasculitis, the probability of anti-HUVEC antibody production was higher than that in healthy subjects (9).

The first aim was to efficiently select EBV-transformed B cells able to produce anti-HUVEC antibodies. In this study, EC-pSV1 cells were used as a source of antigens able to interact with immunoglobulin receptors on the surface of B cells. This human endothelial cell line transfected by SV40 was used for several reasons: (i) the EC-pSV1 cells can be maintained in long-term cultures, (ii) the growth of EC-pSV1 cells is effective without addition of heparin and endothelial cell growth factors, and (iii) the EC-pSV1 cells produce spontaneously high levels of biologically active interleukin-6, which is one of the more effective B cell growth factors (29, 30). In contrast, HUVEC cultures, obtained from different umbilical veins, were chosen as a source of antigen extracts for anti-endothelial antibody screening by ELISA, instead of EC-pSV1 cells, in order to discard the selection of nonrelevant antigenic determinants such as blood group antigens. Human IgM was found not to bind onto HUVEC even at high concentrations. A B cell line (1G5) that produced high levels of IgM did not show any anti-HUVEC antibody-binding activity in comparison with other clones, even with identical levels of IgM (as for the 3G11 B cell line). Thus it is unlikely that IgM antibodies bind through the Fc fragment.

Among the adherent B cells, the frequency of anti-HUVEC antibody-producing B cells increased, in comparison with the level observed with nonselected EBV-transformed B cell populations. Anti-HUVEC antibodies of IgM isotype were clearly detected in supernatants after the ninth day of coculture. The immunoglobulins produced by the selected B cells recognized HUVEC but not MRC5 cells, demonstrating that the augmentation of IgM anti-HUVEC antibody production was not related to an increase of total immunoglobulin production, but to a selection of anti-HUVEC antibody-producing B cells by specific adherence to endothelial cells. Of 21 wells from cloned B cells, 6 demonstrated a strong production of anti-HUVEC antibodies, whereas only 1 of 52 wells from unselected B cells produced low amounts of anti-endothelial cell antibodies. The use of these supernatants on immunoblots against endothelial cell extracts revealed the appearance of 23 bands ranging from 17 to 200 kDa. In contrast, no bands could be detected in supernatants from unselected B cells, even when the coculture supernatants were diluted to adjust the immunoglobulin level to those observed in supernatants of unselected B cells. More interestingly, the frequency of anti-endothelial cell antibody-producing B cell lines was higher in the coculture than in the nonselected culture.

B cell adhesion to endothelial cells involves multiple mechanisms of interaction, including cellular adhesion molecule pathways (intercellular adhesion molecule 1 (ICAM-1)/lymphocyte function-associated antigen 1 (LFA-1) (31–38) and vascular cell adhesion molecule 1 (VCAM-1)/very late antigen 4 (VLA-4) (39)) and antigen–antibody recognition (39–41). VCAM-1 is not spontaneously expressed on both HUVEC and EC-pSV1 cells (42–44), and the fact that B cells adhere strongly in a few minutes to these endothelial cells suggests that in our system, the VCAM-1/VLA-4 interaction pathway was not involved in B cell adhesion. By contrast, adherence of B cells was inhibited by the addition of anti-ICAM-1 antibodies, clearly indicating that this cellular adhesion pathway was involved in the adherence of selected B cells to endothelial cells. This point suggests that a combination of this pathway with the antigen–antibody recognition was implied in the selection by adherence of B cells.

The interaction of B cells with endothelial cells led to their selection, activation, and expansion. The growth of these B cells selected by adherence was found to be dependent on the presence of EC-pSV1 cells, as judged by proliferation, immuno-

globulin production, and anti-HUVEC antibody synthesis. IL-6 is a B cell growth factor produced by EC-pSV1 cells, but also by EBV-infected B cells in an autocrine manner. Thus, we speculated that IL-6 could be involved in the growth of B cells (29, 30). In fact, our data showed that neither soluble EC-pSV1-derived mediators nor rhIL-6 was able to stimulate the growth and the Ig production of selected B cells. These results indicate that cell–cell interactions led to the maintenance of high growth rates and immunoglobulin production for B cells. We cannot exclude the participation of membrane-associated IL-6.

The last point of this study was to define the target antigen recognized by the anti-endothelial cell antibody produced by the cloned B cells selected by adherence. Antibodies produced by the 3G11 and 3G12 lines recognized only a 192-kDa endothelial cell antigen. This antigen was not related to a blood group antigen as it could be sometimes observed. The anti-192-kDa antibodies also recognized antigens in other cellular extracts with different molecular weights. This difference in apparent molecular weight of the antigenic structure recognized by these antibodies may be due to (i) a different glycosylation state or (ii) the recognition of different antigens sharing cross-reactive epitopes. Nevertheless, the characterization of this 192-kDa antigen is now under investigation.

In conclusion, without previous knowledge of the endothelial cell-derived B cell epitopes, we developed a method based on cell–cell adhesion that efficiently selects adherent EBV-infected B cells in cells producing antibodies directed against endothelial cell antigens. In addition, a human antibody of IgM isotype recognizing an endothelial cell antigen with an apparent molecular weight of 192-kDa has been produced under these conditions. This method can represent a powerful tool for investigating the human B cell repertoire in various autoimmune diseases in which the vascular endothelium is thought to play a central role.

ACKNOWLEDGMENTS

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CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY
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Article No. 0057

SHORT ANALYTICAL REVIEW

Selection of Ligands for Polyclonal Antibodies from Random Peptide Libraries: Potential Identification of (Auto)Antigens That May Trigger B and T Cell Responses in Autoimmune Diseases

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The development of random peptide libraries has increased our possibility for analyzing the structural features involved in binding events. Recently, reports have appeared in which these libraries have been successfully used to investigate binding properties of homogeneous proteins such as monoclonal antibodies. However, a more general application of peptide libraries would be the use of polyclonal sera or fluids from patients with autoimmune diseases in biopanning experiments. This would subsequently allow the identification of (auto)antigen leads responsible for the initiation and/or perpetuation of the immune response in these patients. Moreover, the strategy allows the structural characterization of autoantibody specificities in body fluids that have been produced *in vivo* without the introduction of bias due to preferential B cell growth under *in vitro* conditions. The application of this novel strategy for selection of antibody ligands for polyclonal sera as well as to study the nature of immune responses to defined proteins will be discussed with emphasis on the development of peptide reagents for diagnostic and vaccine use.

RANDOM PEPTIDE LIBRARIES

The development and application of peptide libraries have become a powerful tool for identifying determinants recognized by antibodies and other proteins (1–9). Libraries that have been used so far can be divided into three categories that differ in the way in which these libraries were constructed and/or presented (for review see 2). The first category is based on the ability to express foreign peptide on the surface of filamentous bacteriophage, as well the expression of fusion proteins on other vectors such as plasmids (5). The second type involves insoluble, support-bound synthetic chemical libraries in which the amino acids are presented on, for example, resin beads (6). In the third type, the amino acids are synthesized in a manner that permits their use directly in solution (7). All types of libraries involve specific screening and affinity selection of peptides that mimic ligands for particular proteins.

Libraries that utilize filamentous bacteriophages, such as M13, f1, or fd, are generated by chemically synthesizing a random degenerative oligonucleotide with flanking regions that contain sites for restriction enzymes which allow ligation into cloning vectors. The individual peptides are encoded by millions of random oligonucleotides that are expressed at the N-terminus of the minor protein (pIII) at the tip of the phage (8, 9) or over the entire surface of the phage fused with the major coat protein (pVIII) (10, 11). In both cases the random peptides are exposed and are therefore available for selection protocols.

The selection of antibody motifs from phage libraries will depend on the genetic diversity of the library. Such diversity can be examined by analyzing the distribution of bases at each position within the codons via sequencing of random phage clones. To assess more rigorously the diversity of phage libraries, a modified colony hybridization technique has also been developed (12). As an example we have analyzed the data from random sequences of individual clones derived from different phage display libraries (Table 1). The data indicated that all amino acids are represented. However, the frequencies of some amino acids are under- or overestimated in each library, but still range from about 0.5 to 2, consistent with a random distribution of sequences.

To select phages that bind to particular proteins such as monoclonal antibodies, a biopanning technique is used (16). In this procedure the phage library is mixed with a biotin-conjugated ligand. Thus, phages that display appropriate peptides can be immobilized on streptavidin-coated plates, while phage particles that bind weakly or do not bind will be washed away. The bound phages can be eluted unspecifically by treatment with

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low pH or specifically by a competitor (4, 17, 18). The eluted phages are then propagated into *Escherichia coli,* phage particles are prepared, and the same process is repeated to eliminate irrelevant phages or peptides that bind with low affinity. Following screening, the peptide sequences displayed by the selected phages can easily be determined by sequencing the coat protein-encoding region of the phages such as the pVIII gene. Peptides can be displayed as linear or cyclic constraints (Fig. 1).

STRATEGIES USED FOR ENRICHMENT OF POTENTIAL DISEASE-SPECIFIC PEPTIDES

Phage libraries have been used to study antibody–antigen and protein–protein interactions as well as to develop novel antibacterial and biologically active peptides (2–4, 18, 19). However, a more general use of the phage libraries would first be the identification of

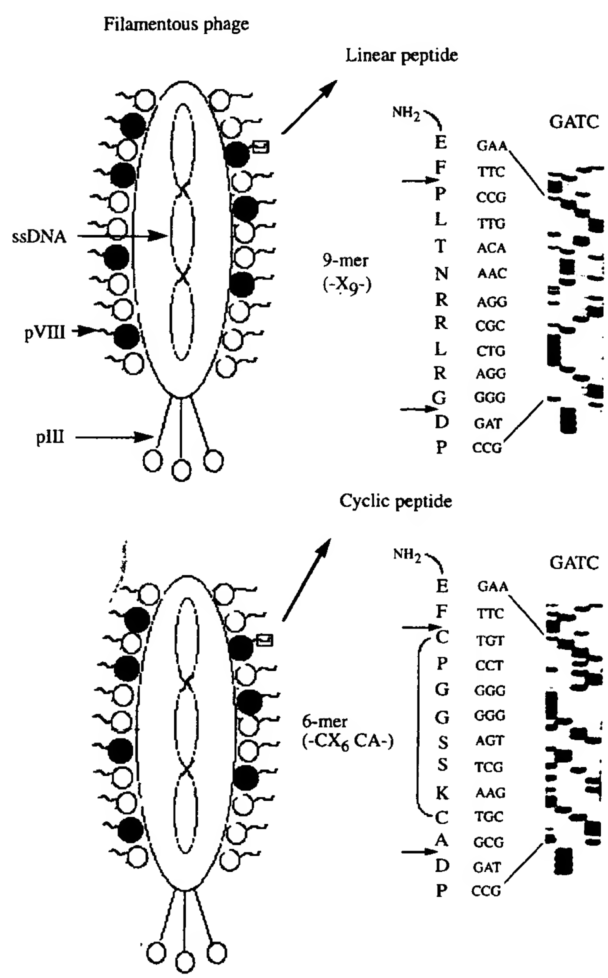

FIG. 1. Shematic representation of peptide displaying phages. Peptides can be displayed as linear or cyclic constraints fused with the N-terminus of the pVIII coat protein on filamentous phages.

TABLE 1

Analysis of the Diversity of Some Random Peptide Phage Libraries

	Occurrence[a]				
Amino acid	Ref. 13	Ref. 14	Ref. 4	Ref. 9	Ref. 15
A	1.29	1.18	1.35	1.42	0.67
C	0.63	0.33	0.47	0.89	0.70
D	0.50	0.66	1.72	1.11	0.73
E	1.50	0.99	1.33	1.00	0.87
F	0.63	0.91	0.55	1.33	1.22
G	2.15	0.81	1.81	1.74	1.67
H	0.38	1.32	1.17	0.78	0.73
I	0.63	1.32	1.56	0.67	0.73
K	1.00	1.74	1.25	1.78	1.16
L	0.84	0.85	0.72	1.25	0.74
M	1.13	0.57	0.47	1.11	0.97
N	0.38	0.99	1.41	0.78	0.73
P	1.38	1.14	0.58	0.47	1.61
Q	1.13	1.57	0.55	1.67	0.58
R	1.32	1.31	0.59	1.04	1.34
S	0.88	0.98	1.08	1.07	1.00
T	0.47	1.22	0.77	0.74	1.10
V	1.41	0.49	1.65	0.95	1.19
W	1.50	0.33	1.17	1.22	1.26
Y	0.63	0.99	0.94	0.67	0.92

[a] The occurrence of each amino acid in different libraries is calculated from the frequency of individual amino acids in random peptide inserts divided by the expected frequency. The expected frequency is calculated by multiplying the fraction of codons which encode each amino acid by the total number of amino acids present in the sample (9).

common features of antibodies present in different individuals infected by the same or related pathogens and second the mapping of polyclonal autoantibodies directed against important functional proteins (e.g., cytokines, snRNPs, DNA, and IgG).

In the first system, the rationale is that antibodies from patient sera might bind to a phage containing the epitope responsible for initiating the disease. Therefore, sequencing of the selected phages may give clues to the nature of the original antigen that initiated the antibody production and possibly the T cell response.

We have been using this approach in order to identify putative antigens that are responsible for the initiation and/or perpetuation of the immune response in patients with autoimmune diseases such as rheumatoid arthritis (RA) (20). However, in contrast to homogeneous proteins such as monoclonal antibodies, the sera from patients or immunized individuals will, in addition to antibodies specific for the antigen, contain a vast array of other antibodies of unknown or irrelevant specificities. This would result in an enrichment of phages with irrelevant peptide ligands during the biopanning procedure. To overcome this problem and to enrich for disease-specific peptides, we have introduced

A

(1) Biopanning with a pool of patient sera (enriched library)
(2) Biopanning of the enriched library with a pool of normal sera (use excess of antibodies compared to phages)
(3) Collection of nonbinder phages, preparation of phages, and screening of the phages by ELISA using a panel of patient and normal sera

B

(1) Biopanning with a pool of patient sera as well as with a pool of normal sera
(2) Subtraction of the two enriched libraries by colony hybridizations
(3) Selection of positive colonies, preparation of phages, and further screening with a panel of patient and normal sera

C

(1) Biopanning with serum or fluid antibodies from a patient
(2) Screening of the enriched library with a second patient and further with a third patient sera or fluid antibodies
(3) Preparation of phages and then screening with a panel of patient and control sera or fluids in ELISA

FIG. 2. Strategies used for enrichment of disease-specific peptides.

a subtraction step in the biopanning protocol (Fig. 2A). In this protocol, the undesirable phages and the phages that bind outside the antigen binding site were subtracted by using a pool of normal sera. Following selection and subsequent subtraction, phages were further selected for their reactivity in ELISA experiments with a panel of RA and normal sera. Phages that react with RA sera but not with normal sera were considered as relevant phages. This strategy has allowed us to select specific peptides using antibodies present in the sera from RA patients without any prior knowledge of the antibodies or proteins involved. The humoral response of RA patients toward the selected peptide was found to be higher compared to the response of controls (20). A data base search with one overselected peptide (pep1, ADGGAQGTA) identified a significant homology with both the cereal glycine-rich cell wall proteins (GRP) and Epstein–Barr virus nuclear antigen-1 (EBNA-1). Antibody activities against synthetic peptides from the EBNA-1 protein have been found to be elevated in certain autoimmune diseases including RA (21). A significant amino acid homology was found between the GRP 1.8 protein, the EBNA-1 protein, and cytokeratins (Fig. 3) (22). Antibodies against a synthetic peptide from the GRP 1.8 protein were also found to be significantly increased in RA patients compared to controls (Dybwad *et al.*, in preparation).

Rheumatoid arthritis is associated with certain HLA types (HLA-DR1 and some HLA-DR4 specificities) (23). We have therefore investigated the possibility of selecting B cell epitopes from the sera of a DR4-positive patient who had a very aggressive form of RA (14). Analysis of peptide sequences displayed by phages selected by using serum from this patient revealed a peptide sequence **RKAL**RLSAS that contains a consensus sequence of XXYY, where X = R/K/H and Y = A/L/I/V, which was also selected by the SF and sera antibodies from other RA patients. The peptide shares 4 amino acids with the peptide sequence ES**RRAL** present in the surface membrane hemolysin of *Proteus mirabilis* (24). The change of R to K represents a conservative substitution. The ESRRAL sequence closely resembles the sequence EQRRAA that is present on both the β chain of HLA-DR1 molecules and on the HLA-DR4 subtypes (Dw14 and Dw15) known to be associated with RA (23, 24). Antibody activity against a 16-amino-acid

```
A
                60        70        80        90        100       110
GRP 1.8 GGGGGGGSGGGGGYAGEHGVVGYGGGSGG-GQGGGVGYGGDQGAGYGGGGGSGGGGGVAY
         :|:|    ::|      :   ::  :|: |: || |:|:|:| :|: ||| |||:|:|||:| |
EBNA-1  SGSGPRHRDGVRRPQKRPSCIGCKGTHGGTGAGAGAGGAGAGGAGAGGAGAGGGAGGAG
        60        70        80        90        100       110

                120       130       140       150       160
        GGGGERGGYGGGQGGGAG--GGYGAGGEHGIGYGGGGGSGAGGGGYNAGAQGGGYGTGG
        |:||: :| |:|:|||||  || ||||: | | |:||:::||:|| :::::||| |:||
        GAGGAGAGGGAGAGGGAGGAGAGGGAGAGGGAGGAGAGGGAGGAGGAGAGGGAGAGG
      120       130       140       150       160       170

      170       180       190       200       210       220
        GAGGGGGGGGDHGGGYGGGQGAGGGAGGGYGGGGEHGGGGGGGQGGGAGGGYGAGGEHGG
        ||||:|:|||: |:| |:|:|:|:||||: |:||: :||:|:| |:|::|| ||||: :|
        GAGGAGAGGGAGGAG-GAGAGGGAGAGGAGGAGAGGAGAGGGAGGAGGAGAGGAGAG
      180       190       200       210       220       230

      230       240       250       260       270       280
        GAGGGQGGGAGGGYGAGGEHGGGAGGGQGGGAGGGYGAGGEHGGGEGGGQGGGAGGGYGA
        |||:| |:||||: |||:: :||||:| :||||:| ||||: :||::||:|:|::|| ||
        GAGAG-GAGAGGAGGAGAGGAGGAGAGGAGGAGAGGGAGGAGAGGGAGGAGAGGAGGAGA
      240       250       260       270       280       290

      290       300       310       320       330       340
        GGEHGGGAGGGQGGGAGGGYGAGGEHGGGGGGGQGGGAGGGYAAVGEHG-GGYGGGQGGG
        ||: |:||||: |:||||| ||||: :|||| |:||::| | :: |::| || || :| |
        GGAGGAGAGGAGGAGAGGGAGAGGAGAGGGRGRGGSGGRGRGGSGGRGRGGSGGRRGRG
        300       310       320       330       340       350

B
                 120       130       140       150       160       170
GRP 1.8  GERGGYGGGQGGGAGGGYGAGGEHGIGYGGGGGSGAGGGGYNAGAQGGGYGTGGGAGGG
                                         |:||::||||| ::|: ||:||:::|:|:
KERATIN  TECQNAEYQQLLDIKTRLENEIQTYRSLLEGEGSSSGGGGGRRGGSGGGSYGGSSGGGSY
                   430       440       450       460       470       480

                 180       190       200       210       220       230
         GGGGGDHGGGYGGGQGAGGGAGGGYGGGGEHGGGGGGGQGGGAGGGYGAGGEHGGGAGGG
         ||::|: ||:|||: |:||: ||| :|||:|||::||| |||:::| |||| |||::|||
         GGSSGG-GGSYGGSSGGGGSYGGGSSGGGSHGGSSGGGYGGGSSSG-GAGG-HGGSSGGG
                   490       500       510       520       530

                 240       250       260       270       280       290
         QGGGAGGGYGAGGEHGGGAGGGQGGGAGGGYGAGGEHGGGEGGGQGGGAGGGYGAGGEHG
          |||:::|   :|::    ::|||: ::  |   |
         YGGGSSSGGQGGSGGFKSSGGGDQSSKGPRY
        540       550       560
```

FIG. 3. Amino acid homology between the GRP 1.8 protein and the EBNA-1 nuclear protein (A) or keratin type 1 cytoskeletal protein (B). :, conserved replacement.

synthetic peptide containing the ESRRAL sequence has been found to be higher in RA patients compared to control groups (25). Another major associated allele, DR4 Dw4, carries the related sequence QKRAA that is present on the gp110 protein of Epstein–Barr virus (26). Furthermore, the QRRAA sequence motif has been described to be a recognition site for alloreactive T cells in RA patients expressing DR1, Dw14, and Dw15 (23).

Taken together, the above examples demonstrate that by using only sera from patients it is possible to select antibody motifs. Such motifs can be used as probes to identify (auto) antigen leads that may play a role in the etiology of rheumatoid arthritis. However, the peptide motifs which we have selected could represent conformational epitopes, since our strategy identifies both continuous and conformation-dependent epitopes (27, 28).

Alternatively, screening of peptide phage libraries with sera from patients who have recovered from diseases may identify peptides for protective antibodies. As mentioned above, patient serum contains millions of different antibody specificities. To increase the possibility of selecting relevant phages, screening of phages with sera from many patients and controls in ELISA experiments is required. To overcome this time-consuming requirement, a rapid immunological screening method has been developed (12). This method allows the direct identification of binding phages following a transfer to nitrocellulose membrane filters. In the past, a similar method has been used in the screening of expression cDNA libraries (29, 30). To improve the screening procedure, in addition to the subtraction step which we introduced in the biopanning experiments (20), we have subtracted two enriched libraries from the screening of sera from RA patients and, in parallel with sera from normal individuals, by colony hybridization (Fig. 2B) in which the probe association is independent of the nature of the sequence. This was accomplished by the addition of 3 *M* tetramethylammonium chloride (TMAC) to both the hybridization and washing buffers (31). Under these conditions the dissociation temperature of AT base pairs is similar to that of GC base pairs. In effect, the binding of a short probe becomes solely dependent on its length.

IDENTIFICATION OF ANTIBODY SPECIFICITIES IN THE SYNOVIUM

For many of the autoantibodies found in the synovium of patients with rheumatic diseases, there is minimal knowledge of what the actual reactive epitopes are and what provided the original stimulus that initiated their production. The localized nature of the immune response in the synovium seen in RA and JRA patients suggests that the synovium compartment may contain or express antigen(s) that are not found in other tissues or they are better presented in the synovium than elsewhere in the body.

Plasma cells in the synovium produce high amounts of immunoglobulins, many of which exhibit rheumatoid factor (RF) activity (32). Locally produced immunoglobulins might play an important role in the pathogenesis of RA by forming immune complexes (33). Thus, the antigen specificities of antibodies in the synovium remain a major scientific question. Antibodies in rheumatoid synovial fluids appeared to react with several proteins obtained from synovial tissue (34). However, the exact nature of these antigenic proteins is unknown. When the suspected parental antigen is known, synthetic peptides have been used to identify linear epitopes recognized by autoantibodies (35). Reactive peptides identified in this way may make better diagnostic tools than the parental protein. Many antibodies, especially autoantibodies, do not recognize linear structures (36), and in those cases the overlapping peptide strategy cannot be applied. The phage library approach gives the possibility of analyzing the structural characteristics of both linear and conformational epitopes. This would allow the identification of antibody specificities found at the inflammatory sites such as in the synovium, regardless of whether the (auto)antigens are known. Another crucial advantage of the phage library is that it permits the identification of autoantibody specificities that have been produced *in vivo* without the introduction of bias due to preferential B cell growth under *in vitro* conditions or potential *in vitro* stimulation of resting B cells by EBV transformation (37). Moreover, polyclonal sera from patients will possess additional specificities that cannot be encountered among monoclonal antibodies. These specificities may have potential significance for the disease (see below).

To begin with, we have screened a nine-amino-acid unconstrained library with polyclonal SF antibodies (14). Phages that share consensus sequences were isolated (e.g., HELGDIAIA, GASGDIAKQ, and APRRLFDIA). High antibody titers against a synthetic peptide, displayed by the overselected phage (HELGDIAIA), were found in other SF samples obtained from RA patients compared to SF samples from osteoarthritis patients ($P < 0.0115$, Mann–Whitney test). Moreover, the humoral response in the sera of RA patients to the same peptide was significantly higher ($P < 0.02$) compared to the response of normal individuals. A homology search with the peptide identified a similar sequence, GELGDIGLP, that is present in collagen IX (residues 682–693). The amino acids LP represent conservative substitutions for IA. This homology is interesting and might be meaningful, since anti-collagen type IX antibodies have been described in RA patients (38).

Sequence data analysis of SF antibodies-selected

peptides indicated that the B cell expansion in the synovium was most likely due to a local and specific antigen stimulation, since most of the SF-selected peptides were different from those selected from the serum of the same patient (14). These findings support the hypothesis that the B cell repertoire in patients with autoimmune diseases is shaped by an antigen-driven response rather than reflecting a polyclonal B cell activation at the site of inflammation (39). In this regard, the analysis of the antibody repertoire expressed in the synovium of a long-standing seropositive RA patient revealed that some V_κ chains were overrepresented in the synovium compared to the blood from the same patient (40). Although the cloning strategy employed by the authors did not permit direct correlation of antibody sequences with antigen specificity, it presented evidence that the expansion of B cells in the synovium is a product of a local antigen receptor-driven selection.

To determine if there are common SF antibody specificities among RA patients, different SF samples were analyzed (14). In this strategy, all selected phages that bound to SF antibodies from one RA patient "enriched library" were screened with SF antibodies obtained from other, different RA patients (Fig. 2C). This protocol allowed us to identify common SF antibody specificities, for example IRRSETPRA and GASGDIAKQ, that contain consensus sequences of (I/L)RR(E/D)X and GDIA, respectively. The former motif was found in type XI collagen (residues 421–425).

To see whether the screening of serial synovial fluid samples taken from the same patient over a period of time by the phage library showed the same focused motifs, a random hexapeptide phage library (8) displayed on pIII was screened by the synovial fluid antibodies from a RA patient obtained on two occasions with a 2-year interval. The sequence displayed by 61 random phages that bound the SF antibodies obtained on the second occasion identified different groups of peptides that showed common motifs (Table 2B). Interestingly, the peptide motifs SGFP(W/L) and LSLY were seen on the first occasion. This would suggest that these specificities may be relevant for the disease.

The most common autoantibodies in RA are RFs, antibodies that bind to the Fc portion of IgG. RFs can comprise up to 25% of synovial immunoglobulin (IgM, IgG, and IgA) in seropositive RA patients (32). Some of the patients which we have analyzed contain a high RF activity in the SF. Thus, the peptide sequences that have been selected from SF with RF activity should contain RF specificities (14). Surprisingly, examination of more than 200 random antibody epitopes did not reveal any significant homology with continuous primary sequences of the Fc fragment of human IgG. These results indicated that the epitopes for RFs are conformation-dependent epitopes. In this regard, previous studies have shown that the determinant recognized by RF was on the Cγ3 domain, and others were identified as belonging to the Cγ2 domain (41). The combination of site-directed mutagenesis and exon exchange used to define the structure of IgG recognized by IgM rheumatoid factors indicated that at least three regions, two from CH2 and one from CH3, contribute significantly to the epitope recognition by monoclonal RFs (42). This would suggest the involvement of the Cγ2 and Cγ3 interface in RF binding. Preliminary analysis of peptide sequences which we have selected by the use human monomeric IgG–agarose affinity-purified polyclonal RFs confirms that RF epitopes are conformation-dependent epitopes. Although the binding of polyclonal RF's to the selected phages was significantly lower as compared to their binding to the Fc of human IgG, the analysis of the sequence data suggested that Tyr, Ser, Leu, His, Phe, Ala, and Arg are important for RF binding. The determination of the antigenic sequences of *in vivo* produced RFs is important in that it may help to identify other antigens responsible for the induction of RF production. In this respect, a homology search with some RF motifs that we have selected identified significant linear homology with human cytoskeletal proteins (e.g., tubulin) and other proteins from viruses that could serve as stimulus to (re)activate the RF-producing B cells. If this proves to be the case, the affinity maturation of RFs will possibly depend upon the specific infection in each individual.

TABLE 2

Peptide Sequences Displayed by 100 Random Phages Binding SF Antibodies from One RA Patient Obtained at the Second Occasion

A			
WWNFGG[6a]	GYRGVN	WWYMGG	VRLNHI
EKVASG[4]	RRQWLT	FYQWMA	KDTFQA
B			
TGFPWA	**LSLYSL**	**LSLLST**	**SPFT**YQ
SGFPWR	**LSLYS**S	**LSLLS**F[2]	LNH**SPF**
I**SGFP**C	**LSLY**FA	GPY**SLL**[2]	**PFSA**SS
PD**SGA**S	**L**QR**YMS**	VT**SFWC**	ACW**PFC**
SLAG**WA**	K**A**H**SL**P	D**L**GR**IS**	**PF**WWLY
QVS**FPW**[2]	**FSL**AWF	**LRLSFT**	**PY**L**AA**F
FPLRYS		**LL**AKNI	**PY**MSNR
A**FPLHF**		P**IIS**QS	
FPLEFW		**II**Y**S**GS	
FPLLFS		MEH**LPS**[6]	
H**FPVSI**		K**S**RR**SS**	
FPGYSL			
F**GF**RNL			

Note. (A) Sequences with no obvious homology to the motifs in B.
[a] The number of clones encoding the same peptide. Motifs are shown in bold.

STUDIES OF B CELL RESPONSES AGAINST IMPORTANT FUNCTIONAL PROTEINS

Sera from patients with autoimmune diseases and some other diseases contain for unknown reasons anti-

bodies against very important functional proteins and cellular components (36, 43). For example, autoantibodies against the small RNA–protein complexes, the small nuclear ribonucleoproteins (snRNPs), are mostly found in the sera from patients with systemic lupus erythematosus (SLE) or SLE-overlaping syndromes (44). Serum antibodies against the p53 tumor-suppressor protein have been found in about 20% of patients with breast, lung, and lymphoreticular cancers (45, 46). In addition, naturally occurring autoantibodies against self-proteins such as lymphokines were found in the sera of patients with autoimmune diseases as well as in the sera of normal individuals (47). To date, very little is known about the nature of B cell responses to these important proteins, the precise antibody epitopes on the parental proteins, and their molecular origin. We believe that the random peptide libraries could be an additional tool for studying B cell responses as well as defining the autoantibody specificities against known proteins.

The structure of peptides that can be selected by the use of polyclonal sera will depend upon the complexity of the humoral response (mono-, oligo-, or polyclonal). As may be expected, a humoral response against a protein will be more heterogeneous in terms of antibody specificities than the response against a peptide. To begin with, we have investigated the nature of the rabbit B cell responses against a synthetic peptide (ALWFRNHFVFGGGTKVT) derived from the λ 2^{315} mouse immunoglobulin chain (48). Among the 36 positive phages that reacted significantly with the antiserum compared to normal rabbit serum, 31 clones carried the sequences AVFGGGTKL, PFFGGGSRA, and APTGGSKRT that were homologous with the immunizing peptide (13). Interestingly, the peptide sequences displayed by the positive clones suggested that the B cell response was focused on the carboxy terminus of the peptide and was of monoclonal type, since the phages shared the same consensus sequence. Thus, these data open the possibility of studying the nature of B cell response against antigens.

The level of naturally occurring autoantibodies to tumor necrosis factor α (TNFα) is significantly increased in patients suffering from chronic infections and various inflammatory disorders, including rheumatic diseases (47). Interestingly, a mouse transgenic for the human TNFα gene, which expressed a high level of the protein *in vivo,* developed chronic arthritis (49). The disease in the mouse could be prevented by administration of mAb against TNFα. Furthermore, it was demonstrated that anti-TNFα antibodies were safe and a benefit for arthritis patients (50). Thus, it is possible to speculate that the *in vivo* stimulation of TNFα autoantibody production may block the effect of the proinflammatory cytokines. Having this idea in mind, as a first step we have tried to determine the specificities of affinity-purified polyclonal autoantibodies against TNFα protein from one RA patient (51). The amino acid composition displayed by the phages that were more frequently selected during the screening experiments revealed a consensus sequence of Ser-Ser-X, where X = Pro, Leu, or Phe. Analysis of the primary structure of TNFα protein indicated that the sequences that we have selected represent conformation-dependent epitopes. However, two of the selected epitopes shared a linear homology with the TNFα protein. One peptide contained Ala-X-X-Leu-Leu-Ala corresponding to amino acids 33 and 36–38 in the TNFα protein, while another peptide contained Pro-X-X-Lys-Pro-Val corresponding to residues 8 and 11–13 in the TNFα protein (52). Since the phages make good carriers for producing antibodies against their displayed peptides, the TNFα-selected phages were used as immunogens. The sera of mice (BALB/c) immunized with phages displaying ASSLLASSP and NSSPYLNTK peptides that contain the consensus sequence showed an anti-TNFα response. Thus, these data provide some measure of significance of the selected peptides. Moreover, it also opens the possibility of defining epitopes that could be used for immunization.

The selection of B cell epitopes from polyclonal antibodies has been successfully demonstrated by other studies (53, 54). Kay *et al.* described the mapping of a polyclonal goat anti-mouse IgG Fc antibody with use of a 38-amino-acid peptide phage library (53). Peptides with a consensus sequence (RT(I/L)(S/T)KP) were selected, and this sequence was present within the Fc region of the mouse Ig (aa 216–221). In addition, a phage expressing a completely different peptide, with no homology to the mouse Ig sequence, was selected. This peptide probably represented a mimotope of a conformational epitope. Recently Folgori *et al.* (54) selected two mimotopes (CRTCAHPGEHA and CGPFFLAASVC) from the screening with sera of individuals immunized with the hepatitis B virus envelope protein (HBsAg). Only the first peptide revealed some homology with amino acids 121–127 of the HBsAg, yet the humoral response to these mimotopes was found to be widespread in the recombinant HBsAg-immunized population (80%).

CAN THE SELECTED PEPTIDES FROM THE PHAGE LIBRARIES INDUCE A HUMORAL RESPONSE?

Production of antisera to a peptide usually involves the chemical synthesis of the peptide and its conjugation to a carrier molecule. However, De la Cruz *et al.* (55) demonstrated that recombinant phages with peptide epitopes displayed on the coat proteins are both antigenic and immunogenic in different animals. This system appeared to be an ideal immunological tool, and many investigators have tested whether the peptides

selected by the phage libraries could induce humoral responses in different animal species. As mentioned above, the peptides that have been selected by their capacity to bind autoantibodies against tumor necrosis factors induced a humoral response in BALB/c mice (51). The HBsAg mimotopes selected from the sera of immunized individuals induced a strong and specific response in mice, rats, and rabbits (54). Recently, Keller *et al.* (56) selected from a 15-residue epitope library peptides that mimic the V3 loop of HIV gp120. None of the 55 selected peptides were absolutely identical to any known HIV-1 variant V3 domain, yet sera from a positive rabbit immunized with one of the selected peptides (LLRTIMIGPGRLLHS) neutralized the HIV-1 variant SF-2 *in vitro*. In contrast to the above results Felici *et al.* (57) demonstrated that even if the selected phages were able to mimic the parental epitope for a mAb, they were unable to elicit a detectable production of antibodies against the original antigen. This could be related to the nature of the peptides tested. It has been found that identical oligopeptides can exist in different conformational forms (58).

PHAGE LIBRARIES AS A TOOL FOR THE IDENTIFICATION OF T CELL EPITOPES

Although autoantibodies may play an important role in a number of autoimmune disorders such as Graves disease and insulin-dependent diabetes, their role in many other autoimmune diseases is not clear. In a number of experimental models of autoimmunity, T cells, not autoantibodies, are able to transfer the disease, indicating a crucial role for T cells in autoimmunity (for review see 59).

In contrast to B cells, T cells recognize peptide fragments presented on the antigen-presenting cells (APC) in the context of either class I or class II MHC molecules. Thus, peptides displayed by the phages cannot be directly used to stimulate T cells, except for peptides that are able to mimic superantigen activities. To be available for APCs, the peptides must be detached from the phages or the solid phase.

To overcome the above problems, a procedure for the detachment of peptides from their solid phase after their simultaneous synthesis by the automated Pepscan method has been developed and used for the localization of T cell epitopes in known proteins (60, 61). Recently, Hickling *et al.* (62) described a simple method of screening recombinant random proteins or peptides for their ability to stimulate T cell clones. By this method single microcultures of *E. coli* each expressing a random peptide sequence fused with protein A were cultured in 96-well plates. Following lysis of cells, each recombinant protein A was captured with immobilized immunoglobulin in tissue culture wells. T cells and antigen-presenting cells could then be added directly to the wells and assayed for proliferation to each individual captured random peptide.

In contrast to antibodies where it is possible to screen millions of specificities in one single experiment, the strategy for T cells is limited, since it requires the mobilization of each recombinant protein displayed by each clone. This may constitute a limiting factor for the application of the phage libraries. Given the greater ease by which B cell antigens can be identified, further studies of antibody epitopes might allow identification of the elusive T cell epitopes responsible for T cell activation. Alternatively, antibody epitopes could be used as a probe for the identification of antigen leads. In this regard, a nanopeptide selected from the phage library by screening RA sera was used as probe to identify the cereal GRPs which are ubiquitous proteins found at varying concentrations in the cell wall of grains and legumes. The examination of the amino acid sequence of the GRP 1.8 protein showed the presence of many glycine/alanine repeated sequences similar to EBNA-1 and cytokeratin (Fig. 3). To investigate a potential T cell response to GRP 1.8 protein, a 15-amino-acid peptide, corresponding to positions 418–432 in GRP 1.8 protein, was made and its ability to stimulate SF T cells from RA and JRA patients was investigated (22). Interestingly, a $DR8^+$ JRA patient with the pauciarticular form showed a strong SF T cell response to the peptide. Sequence analysis of the TcR genes used by the GRP peptide-specific T cell line, the T cell clones, and freshly isolated T cells from this JRA patient identified the same TcR gene usage ($V\beta5.5/D\beta1.1/J\beta2.7$). This emphasizes the monoclonality of these particular T cell subsets in the synovium of this particular JRA patient. This finding suggested that an autoantigen similar to the GRP peptide must be present in the synovium. According to the molecular mimicry hypothesis, a cross-reactive epitope between the host and foreigner proteins may induce autoaggression by evoking autoreactive T and B cells. In this situation the SF T cell clone could be derived from the gut following stimulation with GRP or related proteins. Immunological hyperresponsiveness of the gut mucosa to luminal antigens may subsequently produce sufficient stimuli to perpetuate joint diseases. Our results may clarify the proposed link between gut involvement in inflammatory joint diseases (63).

SOME LIMITATIONS OF THE PHAGE DISPLAY LIBRARIES

Biologic synthetic methods that involve the use of coliphage and *E. coli* are limited by the codon usage bias. The redundancy of the genetic code results in a variation in representation. Such variations may or may not be important, depending on the affinity and specificity of the binding interactions involved between

the ligands and their receptors. For instance, no peptide motifs could be selected from the phage epitope library for six of seven known monoclonal antibodies against keratin 8 (64). A similar low success with monoclonal antibodies has been reported by others (3, 65). Using antibodies from the cerebrospinal fluid of a multiple sclerosis patient, we are unable to select a significant number of epitopes compared to the unselected nanopeptide phage library (Dybwad, unpublished results). This failure may be due to the absence of the antibody epitopes in the libraries tested. This may also arise from difficulties in efficiently preparing and subsequently introducing the library into host strains. Some antibodies may recognize a conformation-dependent epitope, which the library cannot mimic. One solution to these problems could be resolved by the use of constrained libraries, in which peptides are displayed as cyclic constraints (see Fig. 1). Data from many groups indicated that the use of constrained random peptides confers additional advantages by restricting the random peptides to fewer conformations (2, 18). Furthermore, cyclic conformational constraints can be used to increase the affinity of phage-bound peptides. In the future, phage display libraries of different designs may provide a richer source of ligand structures as well as increasing our chance of finding effective mimics of natural ligands.

CONCLUSION

The data discussed here suggest that random peptide libraries can be a useful tool for screening polyclonal sera for motifs binding to *in vivo* produced antibodies. Such motifs can be used either as single or in combination to develop vaccine leads and diagnostic reagents. Moreover, with regard to autoimmunity, antibody motifs can be used as probes for the identification of potential T cell antigen leads. Thus, this novel strategy can give clues to the nature of the etiological agent that may be responsible for the induction and/or perpetuation of autoimmune diseases.

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AB Lymphocytes from patients with HLA class II-linked autoimmune diseases such as type I diabetes, **systemic lupus** erythematosus, rheumatoid arthritis and Graves' have recently been shown to have a decrease in the expression of self-peptide-filled HLA class I antigens on the surface of peripheral lymphocytes. The human demyelinating diseases of multiple sclerosis in some cases are also assocd. with the presence of certain HLA class II genes, which may in turn be linked to genes in the class II region that control class I expression. Hence, we studied fresh peripheral blood

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Reduced Expression of Peptide-loaded HLA Class I Molecules on Multiple Sclerosis Lymphocytes

Fangqin Li, PhD,* Mercedes J. Linan, MD,* Marion C. Stein, MD,† and Denise L. Faustman, MD, PhD*

Lymphocytes from patients with HLA class II–linked autoimmune diseases such as type I diabetes, systemic lupus erythematosus, rheumatoid arthritis, and Graves' have recently been shown to have a decrease in the expression of self-peptide–filled HLA class I antigens on the surface of peripheral lymphocytes. The human demyelinating diseases of multiple sclerosis in some cases are also associated with the presence of certain HLA class II genes, which may in turn be linked to genes in the class II region that control class I expression. Hence, we studied fresh peripheral blood mononuclear cells (PBMCs) and newly produced Epstein-Barr virus (EBV)-transformed cell lines from multiple sclerosis patients for the class I defect. Unseparated PBMCs, as well as T cells, B cells, and macrophages from multiple sclerosis patients had a decrease in the amount of conformationally correct peptide-filled HLA class I molecules on the cell surface compared with matched controls detectable by flow cytometry. To demonstrate the independence of this defect from exogenous serum factors, newly produced EBV-transformed cell lines from B cells of patients with multiple sclerosis maintained the defect. In addition, DR2 +/+, +/−, and −/− EBV–transformed B cells from these patients similarly demonstrated the self-antigen presentation defect. Analysis of a set of discordant multiple sclerosis twins revealed the class I defect was exclusively found on the affected twin lymphocytes, suggesting a role of this class I complex in disease expression. These data indicate that multiple sclerosis patients have abnormal presentation of self-antigens. This phenomenon, common to a number of HLA-linked autoimmune disorders, may be associated with failed self-tolerance and improper T-cell education secondary to faulty HLA class I assembly controlled by HLA class II linked genes.

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Multiple sclerosis (MS) is an autoimmune disease characterized by multifocal, recurrent, or progressive demyelination of white matter of the brain, optic nerve, and spinal cord. As is the case for many autoimmune diseases, MS is more frequent in women, tends to be inherited in some families, and displays genetic associations with certain HLA (human lymphocyte antigen) class II haplotypes. The at-risk alleles vary to some degree between populations. HLA class II Dw2 and DR2 are found in approximately 65% of individuals with MS, compared with 15% of controls [1–3].

Recent studies of other HLA class II–linked autoimmune diseases, including insulin-dependent (type I) diabetes, rheumatoid arthritis, systemic lupus erythematosus, as well as Sjögren's disease, Graves' disease, and Hashimoto's thyroiditis, have revealed a reduced density of conformationally normal HLA class I molecules on the surface of antigen-presenting cells [4]. Conformationally normal HLA class I molecules refers to those class I molecules in which endogenous peptide fragments are present in the peptide groove and are thereby presented to CD8$^+$ T cells. The genes that encode the transport proteins are required for self-peptide presentation, are located in the HLA class II region of the genome, and are now commonly referred to as *Tap-1* and *Tap-2* in higher mammalian species. *Tap-1* and *Tap-2* proteins are believed to form a dimer that transports small endogenous peptide fragments from the cytoplasm to the lumen of the endoplasmic reticulum where they associate with class I molecules. Class I molecules with bound peptide fragments are then rapidly transported to the cell surface, where they are stably expressed. A growing literature suggests that in addition to the traditional role of class I molecules presenting viral fragments for cytotoxic T-cell lysis, class I presentation of endogenous self-antigens may be critical for education of T-cell tolerance to self [5–9]. Therefore, if this pathway represents one route to self-

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tolerance, interruption might contribute to autoreactivity as previously demonstrated for murine and human models of type I diabetes, as well as in transgeneic mouse models with ablated class I expression [5, 6, 10].

In type I diabetes there is a defect in the autologous mixed lymphocyte reaction (AMLR), an in vitro immunological assay that represents the T-cell response to autologous antigen presentation. This defect is characterized by normal $CD4^+$ T-cell proliferation followed by faulty $CD8^+$ T-cell proliferation to autologous antigen presentation [5]. The defective AMLR in type I diabetes is secondary to an antigen-presenting cell defect; it does not represent a primary T-cell defect [5]. This antigen-presenting cell defect appears in part to result from a failure to present self-antigens in the groove of HLA class I molecules. In the NOD (nonobese diabetic) mouse model of type I diabetes, this defect is associated not only with low and unstable surface major histocompatibility complex (MHC) class I expression, a sign of peptide-free class I molecules, but also with a rare *Tap-1* allele that results in lowered levels of *Tap-1* mRNA and protein expression. Furthermore, only humans and mice with interruption of class I progress to diabetes [5, 11].

Because of the similarity of MS to other autoimmune diseases suggested by an old but almost uniform literature demonstrating defective AMLR in lymphocytes from individuals with MS, as well as the literature documenting HLA linkage to the class II region, we decided to extend our studies to MS [12–14]. We have therefore investigated the extent of self-peptide–loaded HLA class I expression on both fresh peripheral blood mononuclear cells (PBMCs) and freshly prepared Epstein-Barr virus (EBV)-transformed B cell lines from individuals with MS.

Patients and Methods

Patients

Patients with MS were identified from the outpatient Neurology Clinic of the Massachusetts General Hospital. Histories and repeated physical examinations were consistent with the diagnosis of MS [15]. Additional patients were excluded if they had a history of other autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, type I diabetes, Graves', Hashimoto's thyroiditis, and Sjögren's disease. Age-matched, healthy individuals served as controls. We established negative family and personal history of autoimmune disease. All fresh blood samples from patients and controls were obtained at a time of no exogenous drug therapy, and no patient had received steroids for 6 months. All blood drawing was performed with full consent; this study was approved by the Human Studies Committee of the Massachusetts General Hospital. A total of 52 MS patients and 52 controls composed the study populations. Not all patients were analyzed for both class I density on peripheral blood lymphocyte (PBL) and EBV cell lines. For neurologic disease control patients we studied the HLA class I density on patients with chronic subdural hematoma (n = 2), chronic hydrocephalus (n = 3), epilepsy (n = 2), and progressive multifocal leukoencephalopathy (n = 1) compared with additional controls (n = 8).

Study Design

Our previous studies revealed some age-related but no sex or HLA class I allele effects on the density of HLA class I determinants on PBMCs from individuals 25 to 55 years old. A comparison of class I density by flow cytometry between control lymphocytes analyzed the same day revealed a less than 1% difference in the percentage of HLA class I cells as well as a less than 2% difference in mean antigen density [4]. However, because flow cytometry demonstrates day-to-day variability, that is, an analysis day effect, patients were always studied the same day as a paired control. An analysis of covariance with terms for age, sex, group, HLA type, and analysis day revealed only a significant effect of analysis day ($p = 0.0004$). These results dictated the study design of paired samples. Data were analyzed with Student's paired t test and are reported as means ± SEM unless otherwise specified. The specimens were analyzed on the flow cytometer in a blinded fashion by the operator.

FLOW CYTOMETRY ANALYSIS. As previously described, careful antibody dilutions, rapid phenotyping, and cell counting are critical for the detection of differences in HLA class I density [16]. Cells must not be exposed to room temperature for greater than 4 hours because the rapid turnover of empty HLA class I proteins can be slowed, thus resulting in the disappearance of the defect. Culture conditions must also be carefully chosen to prevent inadvertently filling of the empty class I grooves with serum containing peptides [16]. PBMCs were immediately prepared from freshly drawn blood and analyzed immediately; a portion of the blood also was used to prepare EBV-transformed B cells. Approximately 2 to 3 months after EBV transformation, the transformed cells were similarly analyzed with HLA class I–specific antibodies, with random pairing to newly transformed control EBV-transformed B-cell lines (n = 37 pairs). In addition, 24 established EBV-transformed B-cell lines from MS patients also were similarly analyzed.

A well-characterized peptide-dependent HLA class I–specific antibody was used for the analysis of conformationally normal class I molecules on the surface of MS lymphocytes. This antibody, W6/32 (American Type Culture Collection, Rockville, MD), recognizes all HLA class I alleles. As described previously, for PBMC analysis 100 μl of a fresh stock of W6/32 antibody solution of 1.6 μg/ml were added to 10^6 cells; alternatively 100 μl of a 0.8 μg/ml solution were added to 10^6 EBV-transformed B cells [4]. This amount of antibody represents an excess over the 50% saturation point and minimizes low-affinity binding to peptide-filled class I molecules. This concentration eliminates irrelevant Fc receptor binding when used at a higher concentration. The W6/32 antibody was generally used in combination with a secondary antibody, FITC (fluorescein isothiocyanate)-conjugated goat antibody to mouse IgG (immunoglobulin G) 2a (Tago, Burlington, CA), for indirect immunofluorescence. In double-color analysis, W6/32 antibody

was directly conjugated to fluorescein isothiocyanate. Background immunofluorescence was determined by an isotype-matched antibody. The value in all cases was less than 5%. Protocols for the class I phenotyping methods are available from the authors.

SUBSET ANALYSIS. All phenotyping was performed on a flow cytometer (Epics Elite, Coulter, New York, NY) with a minimum of 5,000 cells per sample and a sample flow rate of 350 cells per minute. Other control antibodies used in analysis included KC56 (T200), an antibody that reacts exclusively with lymphocytes as well as CD19 (B4), CD11b (MO1), and CD3, antibodies that detect B cells, macrophages–monocytes, and T cells, respectively (Coulter Corp, Hialeah, FL). No differences in epitope densities were observed for these cellular subsets on fresh PBMCs or EBV-transformed B cells. For EBV cells, the density of B4 served as a control marker. The fluorescence-activated cell sorter (FACS) gate for EBV cells is composed of two peaks on forward angle analysis versus side scatter. The upper peak represents the healthier EBV cells and was the exclusive gate reported in this study. For PBL, small nonlymphocyte debris was excluded using a T200 antibody. In addition, viable cells were identified by forward light scatter intensity and exclusions of propidium iodine, a label of dead cells.

As mentioned above, all data were obtained from a Coulter brand flow cytometer. This machine acquires data in log fluorescence intensity. All flow unit numbers are presented in this fashion throughout the text.

PBMCs were also analyzed for the density of HLA class I molecules on T cells, B cells, and macrophages–monocytes by two-color immunofluorescence with directly conjugated W6/32, CD3, B4, and MO1. An open flow cytometry gate excluding only nonlymphocyte debris was used. For two-color analysis, FITC emission was separated from phycoerythrin (PE) emission using appropriate filters, differentially conjugated beads, as well as a control sample labeled with CD4 and CD8 for the determination of the appropriate compensation.

Results

Fresh PBMCs

Fresh PBMCs from MS patients (n = 17) were compared with those from matched random controls (n = 17) prepared on the same day. The conformational-dependent anti-HLA class I antibody W6/32 revealed lowered density of self-peptide–loaded class I molecule in the unseparated lymphocytes of 15 of the 17 patients relative to controls (Fig 1, Table 1). The log mean fluorescence of peptide-filled HLA class I on cells of patients with MS was 8.1 ± 0.95; the log mean fluorescence of peptide-filled HLA class I on matched controls was 13.7 ± 1.2 ($p = 0.002$). A lymphocyte subset analysis of the density of class I molecules on the surface of T cells, B cells, and macrophages–monocytes similarly revealed that the defect was apparent on all cellular subsets (see Fig 1). For instance, T cells from MS patients (n = 14) expressed a mean log fluorescence of conformationally correct HLA class I of 8.3 ± 0.95; control cells (n = 14) expressed a mean of 10.9 ± 1.1 ($p = 0.01$). B cells from MS patients (n = 14) had a density of peptide-filled HLA class I molecules of 25.1 ± 4.2; the mean value for B cells from controls (n = 14) was 44.6 ± 8.4 ($p = 0.003$). Similarly, the macrophages and monocytes from MS patients had less expression of peptide-filled class I molecules. The log fluorescence density of conformationally correct HLA class I on macrophages of MS patients (n = 12) was 22.8 ± 3.7; the HLA class I density on control (n = 12) macrophages was 45.6 ± 10.8 ($p = 0.031$). Figure 2 shows the flow histograms for HLA class I on total PBMCs and PBMC subsets from an MS patient and a simultaneously analyzed matched control. The shift in the amount of conformationally normal epitopes on the surface of cells on the axis (log fluorescence) is evident in all lymphocyte subpopulations. A set of identical twins that had been discordant for MS for 20 years also was studied. In all three PBMC subpopulations, the density of conformationally normal HLA class I molecules was reduced in the affected twin. The log mean fluorescence of HLA class I on MS twin T cells was 5 compared with 14 and 12 for the unaffected twin and control, respectively. For B cells, the log mean fluorescence was 36 for the MS twin and 104 and 66 for the unaffected twin and control, respectively. The twins were simultaneously analyzed on two separate occasions with similar results obtained both times.

Fresh lymphocytes from MS patients were compared with controls for other cell surface markers. The density of CD3 (a T-cell marker), CD19 (a B-cell marker), and CD11b (a macrophage–monocyte) cell-specific markers were not statistically different between MS patients and controls. These data, therefore, ruled out a nonspecific problem with surface protein expression on MS cells causing lowered class I density. As an additional control for these experiments, we compared the density of class I on lymphocytes and macrophages from 8 diverse neurologic disease patients with 8 controls. Two of these patients had chronic subdural hematomas, 3 patients had hydrocephalus, 3 patients had epilepsy, and 1 patient had progressive multifocal leukoencephalopathy. For neurologic control patients, the density of class I for PBMC was 14.1 ± 0.9 and for controls was 13.9 ± 2.2 ($p = 0.91$).

EBV-transformed B-Cell Lines

Because unknown serum factors might modify HLA class I expression, in addition to analyzing fresh PBMCs, we also prepared 37 new EBV-transformed B-cell lines from MS patients and analyzed 24 established EBV cell lines from MS patients. The log mean fluorescence of conformationally normal HLA class I molecules on EBV cell lines derived from MS patients and from EBV cell lines from control individuals was

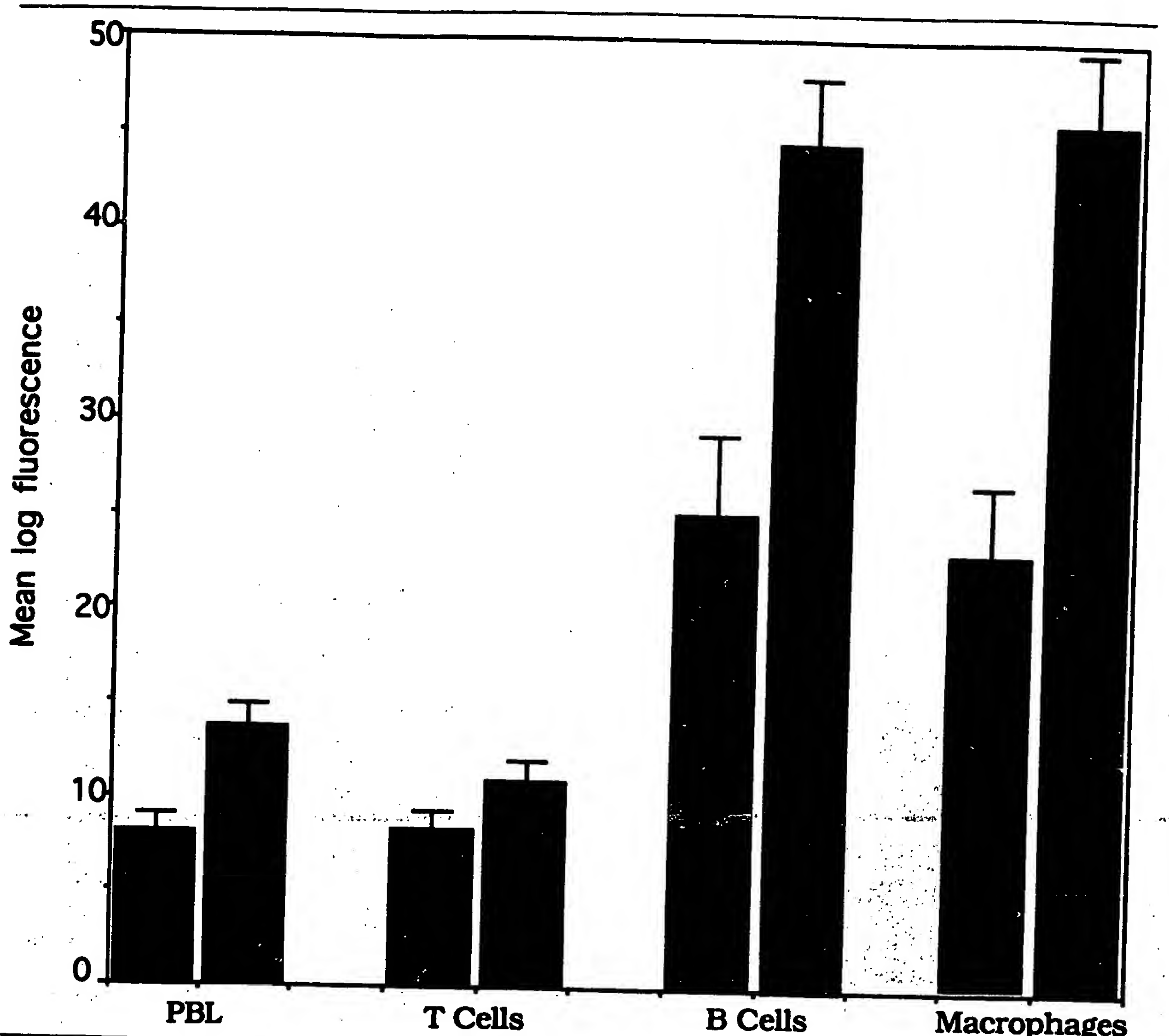

Fig 1. Log mean fluorescence of HLA class I on multiple sclerosis (MS) lymphocyte subpopulation (gray boxes) *compared with age-matched simultaneously studied controls* (black boxes). *Peripheral blood mononuclear cells from 15 of 17 multiple sclerosis (MS) patients had depressed surface HLA class I; T cells from 10 of 14 MS patients had depressed surface class I; B cells from 14 of 14 MS patients had depressed surface HLA class I; macrophages from 10 of 12 MS patients had depressed surface HLA class I.*

5.7 ± 0.4 and 8.0 ± 0.4, respectively (n = 57, matched pairs) ($p < 0.001$). Both established EBV cell lines as well as old EBV cell lines demonstrated the decrease in conformationally correct class I on the cell surface. Many EBV cell lines from MS patients were studied on numerous occasions with different paired EBV cells from control subjects; the decrease in conformationally normal HLA class I molecules in the MS cell lines was consistent. Measurement of the cell surface density of CD19 on MS compared with control

Table 1. HLA Class I Density on the Surface of PBMCs and PBMC Subsets from MS Patients and Controls

	HLA Class I Density (Log Mean Fluorescence)			
Cells	MS Patients	Controls	n	p
All PBMCs	8.1 ± 0.95	13.7 ± 1.2	17	0.002
T cells[a]	8.3 ± 0.95	10.9 ± 1.1	14	0.01
B cells[a]	25.1 ± 4.2	44.6 ± 0.84	14	0.03
Macrophages–monocytes[a]	22.8 ± 3.7	45.6 ± 10.8	12	0.031

[a]T cells were identified with antibodies to CD4 and CD8; B cells were identified with B cell–specific antibody B1; macrophages and monocytes were identified with the specific antibody MO1. All values represent a paired t test of patient compared with simultaneously aged-matched control (standard deviation ± SE).

PBMC = peripheral blood mononuclear cell; MS = multiple sclerosis.

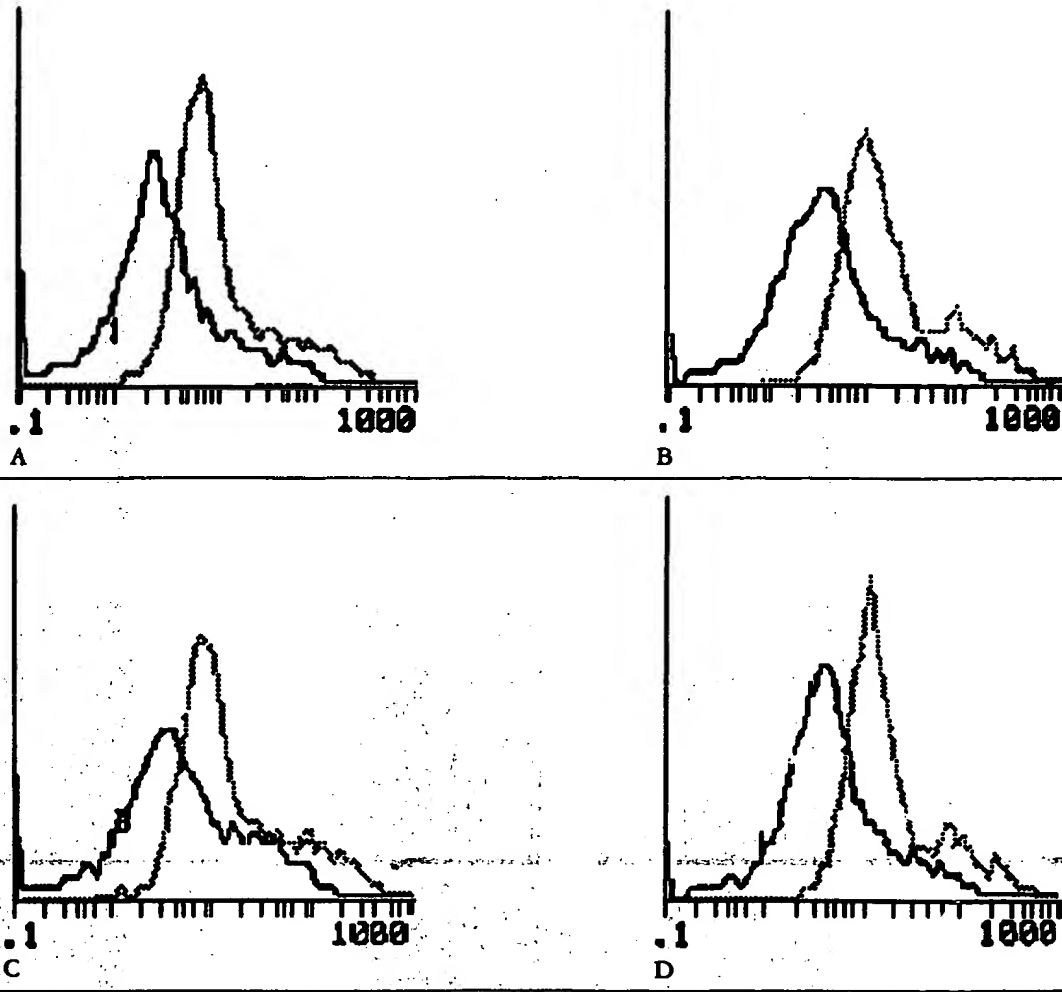

Fig 2. Flow histograms of HLA class I on total peripheral blood mononuclear cells (PBMCs) and PBMC subsets from a multiple sclerosis (MS) patient and a simultaneously analyzed matched control. MS patient (solid) *and a control individual* (dashed). *(A) All PBMCs; (B) T cells; (C) B cells; and (D) macrophages and monocytes.*

EBV cell lines revealed a similar density of the B-cell surface protein (36.5 ± 11 versus 34 ± 9, $p = 0.89$).

Many of the EBV-transformed B-cell lines were analyzed for expression of the common disease-associated HLA DR2 allele. EBV transformed cells from DR2 −/− (n = 30), +/+ (n = 13), and +/− (n = 4) MS patients all demonstrated a similar decrease in HLA class I density compared with HLA-matched control cells (Table 2).

Table 2. Comparison of HLA Class I Density on Newly Prepared EBV-transformed B Cells from MS Patients of Various DR Haplotypes to HLA-matched Paired Controls

	HLA Class I Density (Log Mean Fluorescence)			
DR2	MS Patients	Controls	n	*p*
−/−	6.3 ± 0.6	8.2 ± 0.4	30	<0.01
+/−	4.1 ± 0.8	7.3 ± 1.2	13	0.04
+/+	7.1 ± 0.13	10.4 ± 0.6	4	0.01

EBV = Epstein-Barr virus; MS = multiple sclerosis.

Discussion

Analysis of fresh PBMCs as well as newly prepared EBV-transformed B-cell lines from MS patients revealed a reduced surface expression of conformationally normal HLA class I molecules. The result is similar to our previous data on cells from patients with other HLA class II–linked autoimmune diseases, including Graves' disease, type I diabetes, systemic lupus erythematosus, and rheumatoid arthritis [4]. In addition, fresh blood samples from MS patients revealed that the decrease in conformationally normal HLA class I expression is apparent in T cells, B cells, and macrophages–monocytes. The decrease in HLA class I density observed in both EBV-transformed B-cell lines and fresh PBMCs suggests that exogenous influences, such as serum factors or drugs, are not responsible for

the class I effect in MS patients. A single set of identical twins discordant for MS similarly also demonstrated discordance for decreased expression of class I as is the case for other autoimmune diseases and murine models with variable penetrance of the disease. These data suggest the critical nature of this defect for disease expression. The recent study by Utz and colleagues [17] suggests that changes in T-cell repertoire might be associated with the MS phenotype in discordant twins. Such a result could theoretically arise from differences in HLA class I presentation of self-peptides, which may shape both the CD8 repertoire as well as a subset of CD4 cells [18]. In murine models, it is now established that self-peptides in the groove of MHC class I molecules can select the repertoire of T cells in the thymus as well as the *Tap* alleles determining the peptide repertoire [7, 8, 19].

Familial aggregation in MS, higher concordance between monozygotic than dizygotic twins, and genetic associations with the HLA complex all attest to the strong genetic influence on the penetrance of MS. In whites, DR2, a HLA class II gene, appears to be linked to MS [20]. In other ethnic populations, for example, Arabs [21], Indians [22], Israelis [23], and Japanese [24], DR2 may not play a dominant role. In Italians, DR4 and DR5 are more strongly associated with the disease [25]. In Scotland, MS is most strongly associated with DQw1 [26]. For this study, the penetrance of the decreased density of conformationally correct peptide-filled class I was independent of the DR2 association.

One of the most fascinating unsolved issues in autoimmune research relates to the lack of complete disease penetrance between genetically identical twins and the more frequent occurrence of autoimmunity in females over males. This issue is particularly relevant to the present publication because the class I defect is differentially expressed between discordant MS twins. Does this mean the class I defect is not genetically controlled? Although twins are genetically identical, many genes are differentially expressed between twins; this is especially true for the immune system. Both immunoglobulin gene rearrangement and TCR gene rearrangement represent immunologic diversity generated after conception. This results in divergent immune systems between twins. This was observed by Utz in the T-cell repertoires between discordant MS twins [17]. Furthermore, many genes are allelically expressed; which allele is expressed is determined randomly. Therefore, genetically identical twins can have divergent cellular phenotypes determined by disease-causing genes whose expression is divergently expressed. For example, the variable autoimmune diabetes penetrance in NOD mice, i.e., 85 to 90% of females and 15% males, is controlled, in part, by a sluggish *Tap-1* genotype with sex hormones as well as diverse nonspecific immunostimulatory environmental events altering the genetic defect by up-regulating the inefficient transcription [27]. Similar events may occur in the human, thus helping to explain the incomplete penetrance of MS expression between genetically identical twins without eliminating genetic mechanisms.

Genetic analysis of the HLA region in order to identify the exact genetic defect in MS is difficult because of linkage disequilibrium. For example, congenital adrenal hyperplasia shows a highly statistically significant linkage to the HLA class I allele B47 [28, 29]. However, functional data failed to show a defect in B47 itself but demonstrated instead a deletion in the linked 21-steroid hydroxylase gene. The significant association with B47 is the result of linkage disequilibrium between the 21-steroid hydroxylase and B47 genes, which are separated by over 1,000 kb in the HLA region. Recent genetic evidence has revealed two genes in the HLA class II region that influence class I cell surface density and, more significantly, self-antigen presentation. Because of the instability of HLA class I molecules that are not associated with self-peptide, defects in these genes are easily detected as a decrease in surface HLA class I expression on lymphocytes with conformationally specific class I antibodies. An inspection of the HLA map reveals that these genes referred to as *Tap-1* and *Tap-2* are only 50 kb to the left of HLA class II gene DQ and 200 kb to the right of the HLA class II gene DP. Whether *Tap-2* is linked to the DQ gene in the class II region remains controversial [30, 31].

The proteins encoded by *Tap-1* and *Tap-2* are believed to form an obligatory heterodimer that promotes the delivery of self-peptide fragments into the endoplasmic reticulum, a process that is necessary for the correct assembly of HLA class I molecules. Mutations in either of these two genes may impair HLA class I assembly and will result in accumulation of HLA class I molecules on the endoplasmic reticulum, interruption of self-peptide presentation, and decreased density of conformationally normal HLA class I molecules on the cell surface [16, 32–37]. Furthermore, disruption of the presentation of self-peptides by HLA class I may result in faulty T-cell recognition of self.

To date, three studies have looked for the ability of known *Tap-1* or *Tap-2* polymorphisms to statistically confer MS risk. Statistically significant associations have not been found [38–40]. Although these data may appear to diminish the possible role of *Tap* genes in disease pathogenesis, a number of important reasons could exist for these findings. First, many new polymorphisms within *Tap-1* and *Tap-2* are being reported and these new polymorphisms could confer higher disease risk. Second, all linked mutations or polymorphisms need to be assessed in the context of larger numbers of HLA-matched controls. Since the *Tap-*

determined repertoire selection of self-peptides is relevant in the context of the specific class I alleles, extended HLA haplotype comparisons of patients with controls is also important. Third, MS risk could be caused by the wrong set of normal class I processing genes transporting self-peptides that improperly fit allele-specific class I grooves. We refer to this process as the class I "cassette." This improper allele-specific assembly would be predicted to abnormally select self-reactive T cells. This implies that all seven HLA-linked class I processing genes are important and these diverse genes could represent normal alleles in the wrong context. Statistical studies attempting to find the disease-risk gene would represent the average of multiple genes and localize the defect in the middle of the genetic region. Fourth, the observed class I assembly defects of MS could be controlled by unidentified class I processing genes that are non-HLA linked.

One important question that remains unanswered is why does one individual suffer from MS and another from rheumatoid arthritis if the same self-peptide delivery is interrupted in both instances. Future genetic analysis of *Tap-1* and *Tap-2* will reveal if specific mutations in these genes determine selective mutant self-peptide repertoires and therefore specific autoimmune diseases. Alternatively, non-MHC–linked genes may determine target specificity. In addition, important environmental effects on the penetrance of a disease-associated genotype have been clearly demonstrated in mouse models of autoimmune encephalomyelitis and diabetes [27, 41]. The mechanism for the variable penetrance of diabetes in the NOD mouse appears to be secondary to the effects of many nonspecific stimuli such as dirty mouse rooms, complete Freund's adjuvant, viral infections, bacterial infection, and so on. Many of these factors can prevent diabetes by nonspecifically up-regulating the sluggish *Tap-1* allele production of mRNA, thus restoring class I presentation of self and properly educating $CD8^+$ T cells to self [27]. Therefore, there may be similar immunologic mechanisms operating in autoimmune humans to modify the penetrance of a genetically controlled disease. These issues will be the focus of future studies.

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     antibodies present in lupus sera that are specific for the
     C-terminal region of SmD (sequence 95-119). This region is highly
     homologous to sequence 35-58 of the EBNA I antigen, one of the
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Autoantibodies from patients with systemic lupus erythematosus bind a shared sequence of SmD and Epstein-Barr virus-encoded nuclear antigen EBNA I

SmD is one of the small nuclear ribonucleoproteins frequently targeted by autoantibodies in systemic lupus erythematosus. We isolated and characterized the antibodies present in lupus sera that are specific for the C-terminal region of SmD (sequence 95–119). This region is highly homologous to sequence 35–58 of the EBNA I antigen, one of the nuclear antigens induced by infection with Epstein-Barr virus. Antibodies affinity purified over a peptide 95–119 column were able to recognize this sequence in the context of the whole SmD molecule, as they reacted with blotted recombinant SmD. Anti-SmD 95–119 antibodies bound also the EBNA I 35–58 peptide and detected the EBNA I molecule in a total cell extract from Epstein-Barr virus-infected lines. A population of anti-SmD antibodies is, therefore, able to bind an epitope shared by the autoantigen and the viral antigen EBNA I. To investigate the involvement of this shared epitope in the generation of anti-SmD antibodies, we immunized mice with the EBNA I 35–58 peptide. Sera from immunized animals displayed the same pattern of reactivity of spontaneously produced anti-SmD antibodies. They reacted in fact with the EBNA peptide as well as with SmD 95–119 and recombinant SmD. These data suggest that molecular mimicry may play a role in the induction of anti-SmD autoantibodies.

1 Introduction

Antibodies against small nuclear ribonucleoproteins (snRNP) are frequently produced in systemic autoimmune diseases. Some of these autoantibodies, such as those reactive with proteins associated with U1RNA, are present in a variety of clinical conditions; others, such as the antibodies against snRNP core proteins (or anti-Sm), are serological markers of a specific disease, namely systemic lupus erythematosus (SLE) [1]. In SLE, antibodies against core proteins recognize mainly the polypeptides B, B1 and D [2]. Anti-Sm antibodies are present in 20% of lupus patients, irrespective of their ethnic background and in a very similar proportion of MRL-lpr/lpr mice, one of the lupus-prone mouse strains. The random occurrence of anti-Sm antibodies in genetically different individuals or in inbred populations has raised several questions about the mechanisms involved in their production.

The factors triggering the formation of these autoantibodies are in fact still unknown or debated. Proposed mechanisms include: polyclonal expansion of B cells [3]; cross-reactions with autoantigens of antibodies induced by exogenous antigens [4]; direct triggering of self reactive B cells by autoantigens [5].

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Abbreviations: snRNP: small nuclear ribonucleoproteins EBNA: EBV-encoded nuclear antigen

Key words: Molecular mimicry / Epstein-Barr virus / Autoantibodies / Synthetic peptides

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An effective approach to dissect these mechanisms is to characterize precisely the targets of autoantibodies at the molecular level. The availability of cloned genes encoding several snRNP proteins has allowed the mapping of the antigenic regions on these proteins. Subclones corresponding to different segments of the gene are expressed in bacteria and the recombinant proteins are tested with autoimmune sera. An alternative approach is the use of synthetic peptides; a more accurate mapping of antigenic regions can be obtained by this approach, but only linear epitopes can be mapped.

Among the snRNP proteins, the D polypeptide (SmD) raised our interest because of its strong sequence homology to a viral antigen, EBNA I, induced in the nuclei of cells infected with Epstein-Barr virus (EBV) [6]. The C-terminal region of SmD (homologous to the 35–58 sequence of EBNA I) contains a ninefold Gly-Arg repeat that is responsible for a high hydrophilicity and expression on the surface of the molecule. The antigenicity of this sequence was, therefore, predicted by the authors who cloned and sequenced the SmD protein [6].

We found that the C-terminal portion of SmD is indeed recognized by autoantibodies present in SLE sera. Moreover, we demonstrated that antibodies against the C-terminal peptide react with the homologous EBNA I peptide and with the EBNA I protein and, conversely, that antibodies elicited by immunization with the EBNA I peptide cross-react with SmD.

2 Materials and methods

2.1 Sera

Sera were obtained from patients attending the Clinical Immunology Unit of the University of Pisa and from blood

donors. Diagnosis of SLE, rheumatoid arthritis, Sjogren's syndrome and progressive systemic sclerosis was done according to well-established criteria. Sera from patients with acute infectious mononucleosis were a kind gift of Dr. C. Garzelli (Department of Biomedicine, University of Pisa).

2.2 Immunization of mice

The peptide EBNA I 35–58 was coupled to KLH by 5 mM glutaraldehyde and extensively dialyzed. Five BALB/c mice were immunized at the base of the tail with 25 µg of peptide conjugated to 50 µg of KLH [7] and emulsified in Freund's complete adjuvant. The animals were boosted at 15 day intervals with the same amount in incomplete Freund's adjuvant. Blood samples were obtained from immunized animals 10 days after the second boost. Control sera were obtained from mice immunized with synthetic peptides from *P. berghei* circumsporozoite protein [8].

2.3 Peptide synthesis

Synthetic peptides were obtained by solid-phase synthesis using F-MOC-protected amino acids according to the method of Merrifield as modified by Atherton et al. [9]. The peptides were purified by gel filtration on a Sephadex G-25 column.

GR, GK and peptides contained in the N-terminal portion of SmD were synthesized as linear peptides. GR and GK contain nine repeats of Gly-Arg and Gly-Lys, respectively; N-terminal peptides of SmD correspond to sequences 1–19, 15–34 and 30–49. The C-terminal SmD sequence 95–119 and EBNA I 35–58 (see Fig. 1) have been synthesized as multiple antigen peptides (MAP). A Lys scaffold with two terminal Lys was constructed according to Tam [10] and on the four amino groups of the two terminal Lys four identical sequences were linearly synthesized. An MAP bearing the tetanus toxoid sequence 947–967 [11] was used as a control for direct binding and inhibition assays.

2.4 Detection of anti-peptide antibodies

Peptides were used at a concentration of 10 µg/ml in PBS to coat polystyrene plates (Nunc, Denmark). After blocking for 1 h with 3% BSA in PBS, sera diluted 1/500 in diluting buffer (1% BSA 0.05% Tween in PBS) were added to the plates and incubated for 4 h at room temperature. The plates were washed once with 1% Tween in PBS and twice with PBS. An alkaline phosphatase-conjugated $F(ab')_2$ fragment of goat anti-human IgG (Sigma Chemical Co., St. Louis, MO) in diluting buffer was then added and incubated overnight at 4 °C. After washing (once with PBS-Tween and twice with PBS), the bound enzymic activity was measured with p-nitrophenyl-phosphate. In the case of mouse serum, the bound antibodies were detected by alkaline phosphatase-conjugated goat anti-mouse IgG, IgA, IgM (G-A-M) antibodies (Sigma).

For the competitive assays, 5% dry non-fat milk in PBS was used in the blocking step and PBS 2.5% milk, 0.05% Tween was used as diluting buffer for the sera and labeled antibodies. The amount of serum that gave 50% of the maximum binding was preincubated with different amounts of synthetic peptides or buffer for 1 h at 37° and then transferred to peptide-coated plates. The assay was then carried on as the direct binding assay.

2.5 Isolation of anti-peptide antibodies

SmD 95–119, SmD 30–49 and EBNA I 35–58 were coupled to CNBr-activated Sepharose following the manufacturers' instructions. Briefly, the peptides dissolved in coupling buffer (0.1 M $NaHCO_3$, pH 8.3) were used at 2 mg/ml of gel. After an incubation at 4°, the residual active groups were blocked by 1 M ethanolamine. The sera were absorbed on peptide-coupled Sepharose; the column was washed with PBS until no proteins were detected in the flow through by absorbance at 280 nm. The bound antibodies were eluted by 0.1 M Gly pH 2.8 and dialyzed against PBS.

The immunoglobulin content of eluates and flow throughs from peptide columns was measured by ELISA. Briefly, polystyrene plates were coated with anti-human IgG antibodies (Sigma) diluted 1/1000, blocked with BSA and incubated with eluates and flow throughs at various dilutions. The bound IgG antibodies were detected by addition of alkaline phosphatase-conjugated goat anti-human IgG.

The eluates from SmD 30–49 column never showed any specific peptide-binding activity. Similarly, the eluate of a rheumatoid arthritis serum absorbed on the SmD 95–119 column and the eluate of pooled pre-immune BALB/c serum from the EBNA I 35–58 column did not show any specific peptide-binding activity.

2.6 Anti SmD activity

The recombinant SmD molecule, a kind gift of Dr. Pruuijn and Dr. Van Venrooji, is expressed in the Studier expression system [12] as a fusion protein with 40 added amino acids, encoded by vector and linker. After the induction of expression, bacteria were lysed by repeated freezing-thawing and sonication. The lysate was centrifuged and the pellet containing the SmD protein was dissolved in 8 M urea, 25 mM Tris, pH 7.5. Bacteria transfected with lysozyme were used as negative control.

The bacterial products were separated on a 10–20% acrylamide gradient gel under reducing conditions and transferred to nitrocellulose. The filters were saturated by 1 h incubation in 0.05 M Tris, 0.15 M NaCl and 5% dry non-fat milk. The same buffer was used for antibody dilutions and washings. Purified antibodies at 10 µg/ml or sera diluted 1/500 were incubated on filters for 4 h at room temperature. After repeated washings, alkaline phosphatase-conjugated goat anti-human IgG (Sigma) or goat anti-mouse G-A-M were added and the filters were incubated overnight at 4°. The immunoactive bands were visualized using 5-bromo-4-chloro-indoxyl-phosphate and nitro blue tetrazolium as substrate [13].

For competitive ELISA assays, recombinant SmD was used at a concentration of 1 µg/ml to coat polystyrene plates.

2.7 Anti-EBNA I activity

Paired EBV-negative (BL41) and EBV-converted (E95B BL41 and E95 C BL41) Burkitt lymphoma lines were established as previously described [14]. The DG75 EBV-negative Burkitt lymphoma line and the EBNA 2-transfected DG75 FA were described elsewhere [15, 16].

Total cell extracts were prepared by sonication of 10^7 cells in 1 ml of sample buffer (Tris buffer containing 5% SDS and 5% 2-mercaptoethanol); they were boiled for 5 min, run on a 7.5% acrylamide gel (10^6/lane) and blotted to nitrocellulose. The filters were probed with affinity-purified anti-SmD antibodies and with reference anti-EBNA I antibodies (affinity-purified human antibodies specific for the EBNA I Gly-Ala repeat) [16, 17].

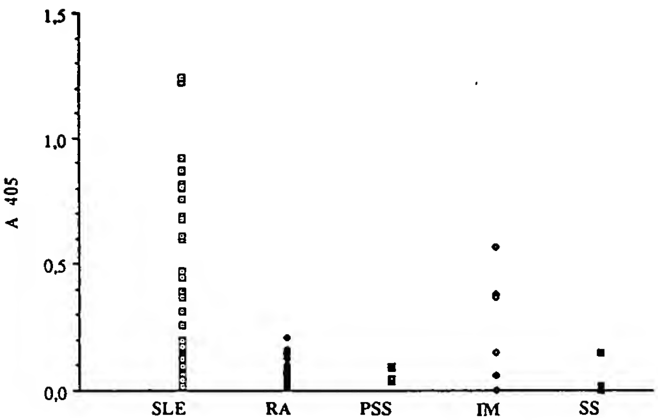

Figure 2. Binding of sera from patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), progressive systemic sclerosis (PSS), infectious mononucleosis (IM) and Sjögren' syndrome (SS) to SmD 95–119 peptide. Sera at 1/500 dilution were incubated on peptide-coated plates and bound IgG antibodies detected by an alkaline phosphatase-labeled anti-human IgG antibodies. Results are expressed as individual sera absorbance at 405 nm 30 min after substrate addition.

3 Results

3.1 Sera reactivity with the C-terminal SmD peptide

To test the reactivity of the C-terminal SmD sequence, a branched peptide that bears four linear 95–119 sequences was synthesized (Fig. 1). The synthetic peptide was used as antigen on the solid phase to measure serum antibodies in an ELISA assay (Fig. 2). Antibodies against the 95–119 peptide were present almost exclusively in SLE (in approximately 30% of the sera) and in a small percentage of infectious mononucleosis sera. Positive results (*i.e.* higher than the mean plus two standard deviations of 30 normal sera) were in fact obtained in 23/63 SLE, 3/23 infectious mononucleosis, 1/23 rheumatoid arthritis, 0/6 progressive systemic sclerosis and 0/7 Sjögren's syndrome sera.

As shown in Fig. 3 (a and b), the specificity of the reaction is confirmed by liquid-phase inhibition: preincubation of sera with the peptide inhibits up to 60% of binding to the solid-phase peptide. Preincubation of serum 2 with the 95–119 peptide inhibited also the binding to solid-phase recombinant SmD (Fig. 3 c). However, only in a few sera (20% of the sera containing antibodies to the 95–119 peptide) the majority of serum anti-SmD antibodies reacts, as in this case, with a linear epitope located in the 95–119 sequence. In most sera, only 20–30% of anti-SmD reactivity is inhibited by the C-terminal peptide. The anti-SmD reactivity not inhibited by the C-terminal peptide can be explained by: (a) binding to linear sequences which we did not synthesize; (b) binding to discontinuous epitopes, *i.e.* sequences of amino acids not contiguous in the primary structure but brought together by the folding of the molecule; (c) binding to conformational epitopes of the protein that cannot be mimicked by synthetic peptides; and (d) binding to novel sequences present only in the recombinant and not in the native protein (that is, encoded by bacterial genes).

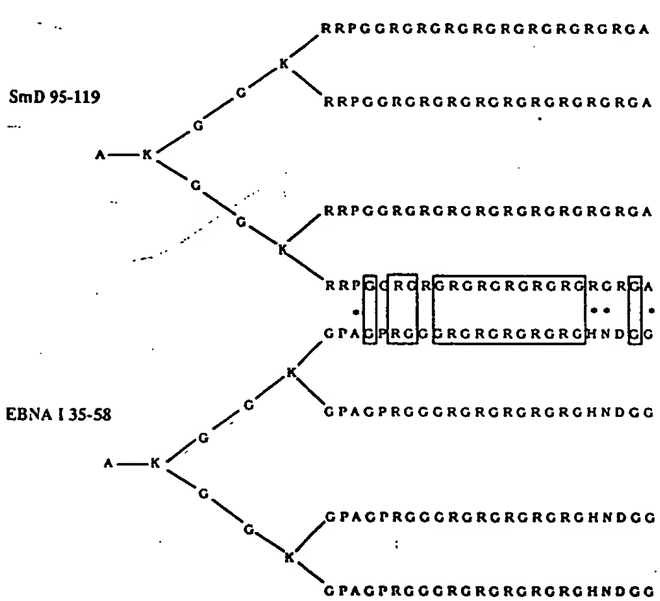

Figure 1. Structure of the multiple antigen peptide SmD 95–119 and EBNA I 35–58. Identical amino acids are enclosed in boxes; conservative changes are indicated by asterisks.

3.2 Specificity of anti-SmD 95–119 antibodies

Anti-peptide antibodies specific for the 95–119 SmD sequence can be purified from SLE sera by affinity chromatography on peptide-coupled Sepharose. Eluted antibodies are able to recognize the sequence on the SmD protein, since on the blotted lysate of transfected bacteria they detect a band of 18 kDa (Fig. 4), corresponding to the molecular weight of the recombinant SmD. The specificity of eluted antibodies from 5 lupus sera was analyzed by an ELISA using different peptides. Anti-peptide antibodies recognize the 95–119 sequence and bind also to the EBNA I 35–58 peptide and to a gly-arg peptide. On the contrary, low binding was detected with a peptide of similar charge (Gly-Lys), indicating that a specific sequence and/or a certain spatial distribution of charges are recognized by the anti-95–119 antibodies (Fig. 5). No binding was detected to a control MAP corresponding to sequence 947–967 of tetanus toxoid (data not shown).

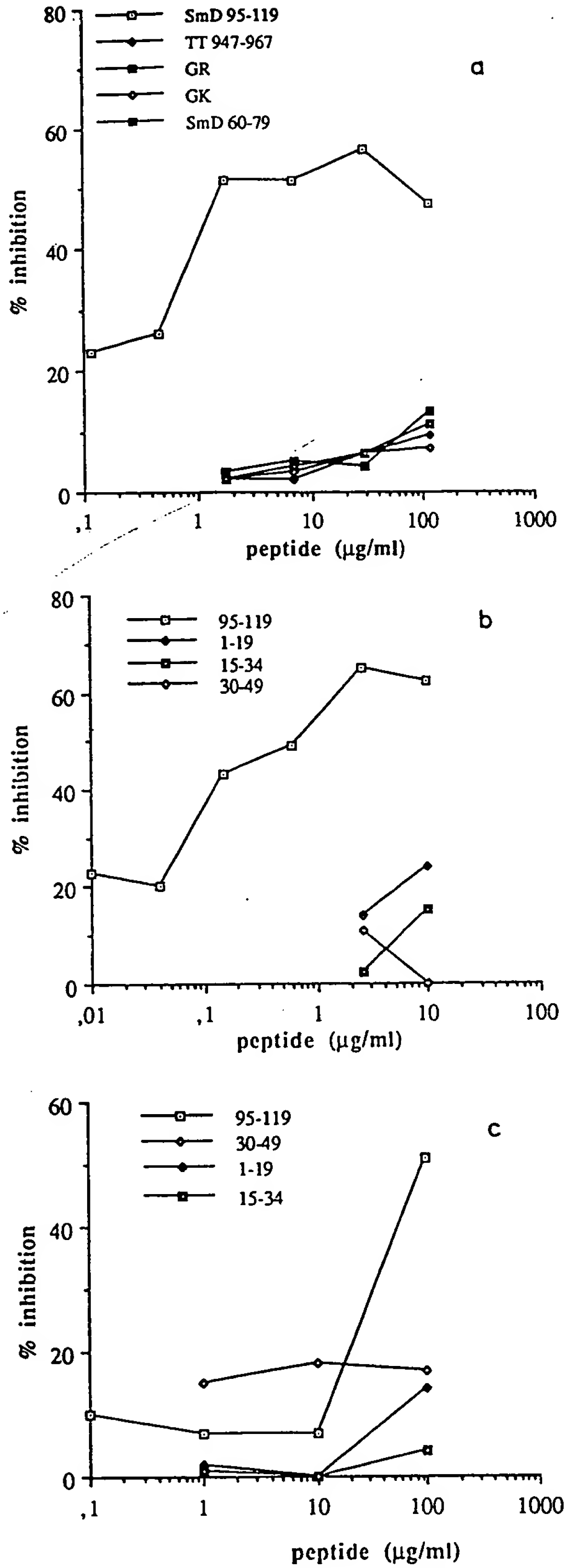

Figure 3. Inhibition of lupus sera binding to SmD 95–119 peptide. Sera were preincubated with different amounts of SmD 95–119, control SmD peptides, GR, GK, TT 947–967 for 1 h at 37 °C and then transferred to 95–119-coated plates [serum 1(a); serum 2(b)] or recombinant SmD-coated plate (c). Bound antibodies were detected as in the direct binding assay. Results are expressed as percent inhibition: 100-absorbance (serum + peptide)/absorbance (serum + buffer).

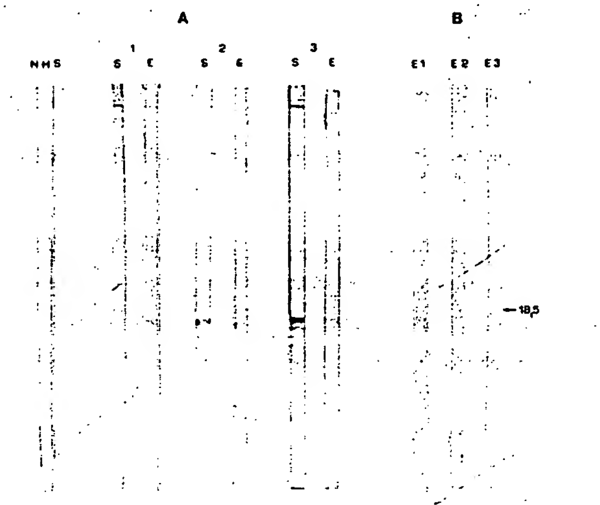

Figure 4. Binding of affinity-purified anti-SmD 95–119 antibodies to recombinant SmD protein. Lysates from bacteria transfected with SmD (A) and from control bacteria (B) were run on a 10–20% SDS gel under reducing conditions and transferred to nitrocellulose. The filters were probed with whole sera (S) or sera eluates from a SmD 95–119 column (E). Three different lupus sera and the corresponding eluates were tested (1, 2, 3). Lupus sera and anti-peptide antibodies detect a band of 18 kDa, that corresponds to the molecular mass of the recombinant SmD molecule; no reactivity is detected with control bacteria that do not express SmD.

The reactivity with the Gly-Arg peptide shows that at least part of the antibodies reacting with both the SmD and the EBNA I peptide recognize the Gly-Arg stretch shared by the two sequences. Peptide binding activity is greatly reduced in serum flow through from the peptide column (Fig. 5).

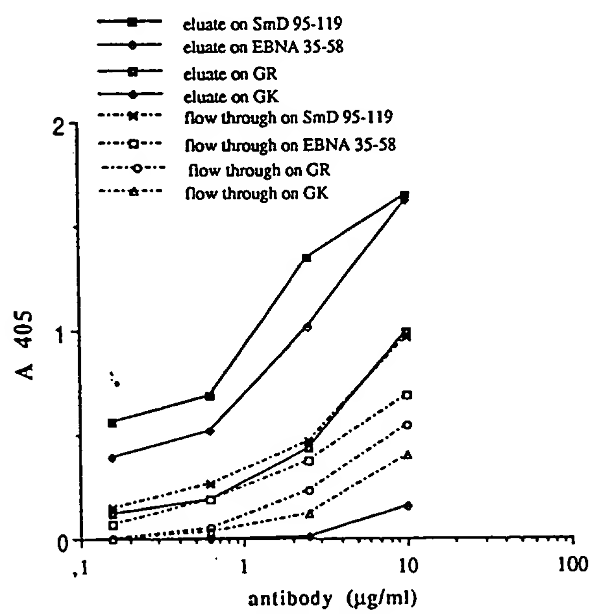

Figure 5. Binding of affinity purified anti-SmD 95–119 antibodies to SmD 95–119, EBNA I 35–58, Gly-Arg and Gly-Lys peptides. Serum 1 eluate (continuous line) and flow through (dotted line) from a SmD 95–119 column were incubated at various concentrations on peptide-coated plates. The bound antibodies were detected by alkaline phosphatase-labeled anti-human IgG. Results are expressed as absorbance at 405 nm 30 min after substrate addition.

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Further evidence for the cross-reactivity of anti-SmD 95–119 antibodies with EBNA I emerged from our analysis of the EBV-infected Burkitt lymphoma lines. As shown in Fig. 6, a band of 76 kDa was detected by anti-SmD and by anti-EBNA I antibodies on the total cell extract from EBNA I-expressing lines. No reactivity was observed with the EBNA I-negative lines.

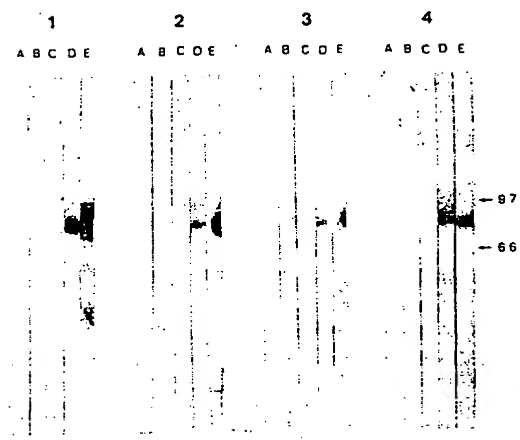

Figure 6. Binding of anti-SmD 95–119 antibodies to EBNA I. Total cell lysate from EBNA I-negative and EBNA I-expressing lines were separated on a 7.5% acrylamide gel under reducing conditions and transferred to nitrocellulose. Nitrocellulose strips were probed with reference anti-EBNA I antibodies (1) or affinity-purified anti-SmD 95–119 antibodies (2, 3, 4). The cell lines used were: (A) DG75; (B) DG75FA; (C) BL41; (D) E95B BL41; (E) E95C BL41. Anti-SmD 95–119 antibodies detect a 76-kDa band present only in EBNA I-expressing lines (lanes D and E).

3.3 Induction of anti-SmD antibodies

The results obtained show that antibodies to the C-terminal portion of the SmD molecule present in SLE sera cross-react with the EBNA I molecule. In the immune response to the EBNA I antigen, anti-SmD antibodies may be generated. To verify this hypothesis, we immunized BALB/c mice with the EBNA I 35–58 peptide in adjuvant. All immunized animals produced antibodies reactive with the immunizing peptide, with the SmD 95–119 sequence and with the Gly-Arg peptide. Anti-EBNA I 35–58 antibodies were purified by affinity chromatography on EBNA I 35–58 coupled to Sepharose. Their binding specificities are shown in Fig. 7. Moreover, the sera and the anti-EBNA I 35–58 antibodies recognized in immunoblot the recombinant SmD protein (Fig. 8).

Immunization with the EBNA I sequence, therefore, induced antibodies which cross-react with the SmD protein.

4 Discussion

The data presented here show that SLE sera contain antibodies to the C-terminal portion of the SmD molecule, which is highly homologous to the 35–58 sequence of the EBNA I antigen. The biological relevance of this homology is outlined by (a) the cross-reactivity of anti-SmD 95–119 antibodies with the 35–58 EBNA I sequence and with the whole EBNA I molecule, and (b) the cross-reactivity of immunization-induced anti-35–58 EBNA I peptide antisera with both the C-terminal SmD peptide and the

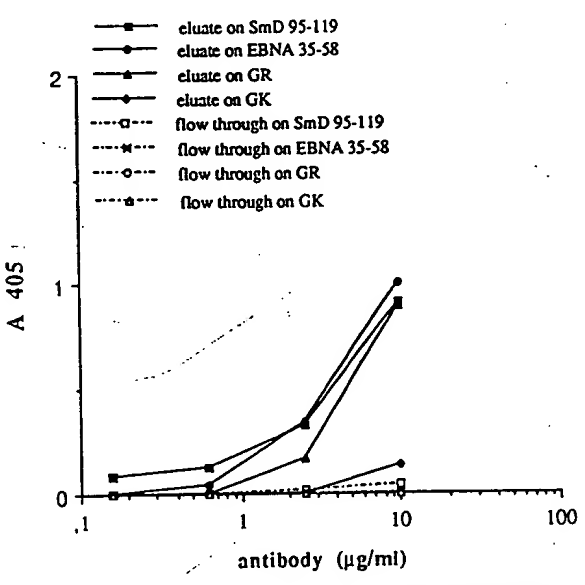

Figure 7. Binding of immunization-induced anti-EBNA I antibodies to synthetic peptides EBNA I 35–58, SmD 95–119, Gly-Arg and Gly-Lys. Anti-EBNA I 35–58 antibodies were purified by affinity chromatography on EBNA I 35–58 column. The eluate (continuous line) and the flow through (dotted line) of the column were incubated at various dilutions on peptide-coated plates. The bound antibodies were detected by addition of alkaline-phosphatase labeled goat anti-mouse G-A-M antibodies. Results are expressed as absorbance at 405 nm 30 min after substrate addition.

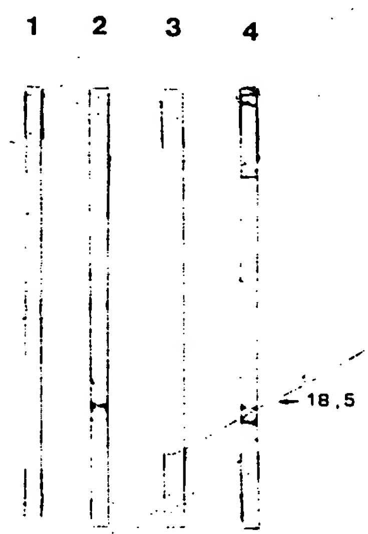

Figure 8. Binding of immunization-induced anti-EBNA I antibodies to recombinant SmD. Lysates from bacteria transfected with SmD were run on a 10–20% SDS gel under reducing conditions and transferred to nitrocellulose. The filters were probed with control serum from a BALB/c mouse immunized with a *P. berghei* peptide (lane 1), with the serum from a mouse immunized with EBNA I 35–58 (lane 2), with the flow through of immune serum from the EBNA I column (lane 3) and with affinity-purified anti-EBNA I antibodies (lane 4). The upper band corresponds to the molecular weight of the recombinant antigen.

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Immunoblotting reactivity of sera from patients with autoimmune connective tissue diseases against Epstein-Barr nuclear antigen (EBNA) polypeptides

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Abstract

The antibody responses to Epstein-Barr nuclear antigen (EBNA) polypeptides were analyzed by immunoblotting in 93 patients with autoimmune connective tissue diseases (ACTD) in comparison with 50 clinically healthy control subjects. Antibody frequencies to EBNA-2, -4, and -6 were significantly higher in patients than in controls. Among the patients with ACTD, those with systemic lupus erythematosus (SLE) showed a significant increase in the frequency of anti-EBNA-3 antibodies. These results confirm the particularity of the antibody responses against Epstein-Barr virus (EBV) polypeptides in patients with ACTD; they could either reflect basic immune disturbances or suggest a participation of EBV in the pathogenesis of the disease.

Keywords: Epstein-Barr virus; EBNA; Antibodies; Connective tissue diseases

1. Introduction

Epstein-Barr virus (EBV) is a human herpesvirus that subclinically infects the majority of individuals. It represents the causative agent of infectious mononucleosis (IM) and is associated with endemic Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC) and other lymphomas in immunodeficiency states. The polypeptide components, as well as the coding regions of most of the EBV antigens originally defined by immunofluorescence, are now well characterized [1]. One of these antigens, the Epstein-Barr nuclear antigen (EBNA) is composed of six polypeptides, namely EBNA-1, EBNA-2, EBNA-3, EBNA-4, EBNA-5, and EBNA-6; alternative designations for EBNA-3, EBNA-4, EBNA-5, and EBNA-6 are EBNA-3A, EBNA-3B, EBNA-LP, and EBNA-3C, respectively [2].

The relationship between EBV infection and autoimmune connective tissue diseases (ACTD) has attracted a great deal of interest. ACTD are characterized by the production of autoantibodies reacting with intracellular proteins and nucleic acids and are associated with anomalies in T cell functions [3]. In previous immunoblotting studies [4,5], we observed that, compared with clinically healthy subjects, patients with ACTD showed significant differences in the prevalence of antibodies to several EBV-early or -structural polypeptides. In the present study, we examined the antibody response against EBNA polypeptides in sera from patients with ACTD.

2. Patients and methods

Sera were obtained from 93 patients. Thirty-three of them had systemic lupus erythematosus (SLE), 21 had mixed connective tissue disease (MCTD), 21 had Sjögren's syndrome (SS); the 18 remaining patients who could not be classified into one of the above diseases were therefore referred as unclassified connective tissue diseases (UCTD). Patients with SLE and SS met the American Rheumatism Association criteria [6,7]. Pa-

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tients with MCTD had typical clinical features such as Raynaud's phenomenon, polyarthralgia, polyarthritis, swollen hands, myositis, and occasionally systemic features as well as high titers of anti-ribonuclear protein (RNP) antibodies confirmed by the Ouchterlony immunodiffusion technique. Anti-RNP, anti-Sm, anti-SSB/La and anti-SSA/Ro specificities, which are considered as biological markers of MCTD, SLE, and SS, respectively [8], were determined by immunoblotting and immunoprecipitation of small nuclear RNAs [9]. Fifty sera from clinically healthy blood donors served as controls. Four sera previously characterized by immunofluorescence on EBNA-1, -2 and -3-transfected cells were used as reference sera: three of them were positive for anti-EBNA antibodies; the other one was anti-EBNA negative. Sera were tested by immunoblotting at a 1:100 dilution.

The EBV-producer B95–8 and P3HR1, as well as the EBV-non-producer Raji, Daudi, and Namalwa cell lines, were used as antigen for identification of the EBNA polypeptides; the EBV genome-negative BJAB cell line served as negative control. Cell culture, cell lysis, electrophoresis and immunoblotting were performed as previously described [4,5]. Antibodies in sera from patients and controls were detected by using Raji and B95–8 cell extracts as antigen. Frequencies of anti-EBNA antibodies in the different groups of patients were compared by using the χ^2 test.

3. Results

Reference sera allowed the identification of five polypeptides with molecular masses of 70–80 kDa, 92 kDa, 140–145 kDa, 150 kDa, and 160–165 kDa (Fig. 1); in accordance with previously reported results, these polypeptides could be identified as EBNA-1, EBNA-2, EBNA-3, EBNA-6 and EBNA-4, respectively [2]. As expected, they were all present in the Namalwa cells which exclusively express the EBV latent antigens; the 92 kDa (EBNA-2) polypeptide could not be detected in P3HR1 and Daudi cells, whereas the 150 kDa (EBNA-6) polypeptide could not be detected in Raji cells [10,11]. None of these polypeptides could be detected in the EBV-negative BJAB cells.

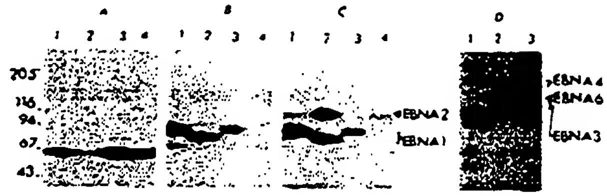

Fig. 1. Identification of the EBNA polypeptides. (a) Anti-EBNA-negative serum: 1, B95–8 cells; 2, P3HR1 cells; 3, Raji cells; 4, BJAB cells. (b) Anti-EBNA-1–positive serum: 1, P3HR1 cells; 2, Raji cells; 3, Namalwa cells; 4, BJAB cells. (c) Anti EBNA-1 + 2-positive serum: 1, P3HR1 cells; 2, Raji cells; 3, Daudi cells; 4, BJAB cells. (d) Anti-EBNA-3 + 4 + 6-positive serum: 1, BJAB cells; 2, B95–8 cells; 3, Raji cells.

Serum reactivities against the five EBNAs analyzed are presented in Table 1. Compared with the controls, a high proportion of patients with ACTD had antibodies against EBNA-2 ($P < 0.001$), EBNA-4 ($P < 0.001$) and EBNA-6 ($P < 0.001$), as well as against all the five EBNA polypeptides ($P < 0.01$). The highest prevalence of these antibodies was observed in sera from patients with SLE. Reactivity against all five EBNA polypeptides was significantly increased in patients with SLE, in comparison with the controls ($P < 0.001$) or the patients with other ACTD ($P < 0.01$). The frequency of

Table 1
Number (%) of sera reacting in immunoblotting with the different EBNA polypeptides in patients with CTD and control individuals

Subjects	EBNA-1	EBNA-2	EBNA-3	EBNA-4	EBNA-6	All five EBNAs
Controls ($n = 50$)	50 (100)	13 (26)	21 (42)	4 (8)	6 (12)	4 (8)
SLE ($n = 33$)	30 (91)	20 (61)	30 (91)[a]	31 (94)[b]	28 (85)	18 (55)[a]
MCTD ($n = 21$)	19 (90)	16 (76)	10 (48)	16 (76)	15 (71)	4 (19)
SS ($n = 21$)	15 (71)	14 (67)	6 (24)	7 (33)	14 (67)	2 (9)
UCTD ($n = 18$)	16 (89)	11 (61)	5 (28)	8 (44)	7 (39)	2 (11)
ACTD ($n = 93$)	80 (86)	61 (66)[c]	51 (55)	62 (67)[c]	64 (69)[c]	26 (28)[d]

[a] Significantly different from controls ($P < 0.001$), MCTD ($P < 0.01$), SS ($P < 0.01$), and UCTD ($P < 0.01$).
[b] Significantly different from controls, SS, and UCTD ($P < 0.001$).
[c] Significantly different from controls ($P < 0.001$).
[d] Significantly different from controls ($P < 0.01$).

anti-EBNA-3 antibodies was significantly increased only in patients with SLE ($P < 0.001$).

4. Discussion

In the present study, we investigated the antibody responses against EBNA polypeptides in patients with autoimmune connective tissue diseases. However, the antibody response against EBNA-5 was not addressed. Indeed, the antibody response to EBNA-5 could not be easily distinguished because this antigen is, in fact, composed of multiple protein species [12] that comigrate with the polypeptide components of the early diffuse antigen (EA-D). These latter components were frequently detected by our sera in B95-8 and P3HR1 cells [5]. Furthermore, EBNA-5 was not easily distinguishable from the latent membrane protein, another EBV-related antigen expressed in the EBV-producer and non-producer cells [13]. For these reasons, our study only focused on the antibody responses to EBNA-1, EBNA-2, EBNA-3, EBNA-4, and EBNA-6.

High prevalences of anti-EBNA antibodies have been reported in EBV-associated diseases such as BL or NPC [13]. Therefore, these antibody responses in ACTD could have pathogenic significance. We have previously reported that patients with ACTD present a peculiar antibody response to EBV-specific polypeptides [4,5]. Indeed, we have observed that sera from patients with ACTD, especially SLE, recognize more frequently the EBV-induced early diffuse polypeptides. Moreover, we also observed that patients with SLE and MCTD present an increased frequency of antibodies against the 42 kDa envelope polypeptide of EBV, as do sera from patients with IM [14]. Therefore, the results obtained in the present study confirm the particular reactivity of sera from patients with ACTD, and especially those with SLE, against EBV antigens. The high frequency of antibodies against EBNA-2, -3, -4 and -6 suggests that the expression of these polypeptides is increased in these patients. Cells expressing all EBNAs and latent membrane protein (LMP) are considered as proliferating cells [15]. These cells, which represent a target for cytotoxic T cells, are continuously destroyed in the normal host [16]. However, in subjects with impaired T cell response such as patients with ACTD, these EBV-activated cells expressing EBNA polypeptides may persist, inducing an increased antibody response.

The particular antibody responses to EBV in our patients could also reflect an increased antigenic stimulation linked to viral replication. Indeed, the defective T cell response associated with ACTD could elicit an increased activity of EBV in these patients. Therefore, given the antigenic cross reactivities and the genomic homologies between EBV and nuclear autoantigen [17–19], as well as the fact that EBV is a polyclonal B cell activator that can induce autoantibody synthesis in vitro [20,21], the involvement of EBV in the pathogenesis of ACTD can be taken into consideration. However, the responsibility of EBV in the development of autoimmune responses in ACTD remains to be established.

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AUTOANTIBODIES FROM PATIENTS WITH SYSTEMIC LUPUS
YTHEMATOSUS BIND A SHARED SEQUENCE OF SMD AND EPSTEIN
ARR VIRUS-ENCODED NUCLEAR ANTIGEN
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Immune Response to Different Sequences of the EBNA I Molecule in Epstein–Barr Virus-Related Disorders and in Autoimmune Diseases

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Epstein–Barr virus (EBV) infection is associated with production of autoantibodies. The N-terminal 35–58 sequence of EBNA I, one of the nuclear antigens encoded by EBV, is highly homologous to the C-terminal 95–119 region of the ribonucleoprotein SmD. Autoantibodies specific for SmD are present only in systemic lupus (SLE) sera and are therefore considered a serological marker of SLE.

We measured antibodies to the EBNA I 35–58 sequence in EBV-related diseases and in autoimmune disorders. Antibodies to the EBNA I 35–58 peptide were present in 30% of normal sera, 12% Burkitt lymphoma, 22% infectious mononucleosis, 25% rheumatoid arthritis, 38% SLE and 33% Sjogren' syndrome. Antibodies to the SmD 95–119 peptide were detectable in 32% of SLE sera, 17% infectious mononucleosis and 12% Burkitt lymphoma. The specificity of anti-EBNA I 35–58 antibodies affinity-purified from nine sera was analysed by means of an inhibition assay. Only anti-EBNA I 35–58 antibodies affinity-purified from SLE sera have a similar affinity for the viral peptide and the SmD C-terminal one; they also bind the recombinant SmD in western blot. The results indicate that antibodies to EBNA I 35–58 are produced in normals, in EBV-related diseases and in autoimmune disorder, but only SLE sera contain anti-viral antibodies cross-reactive with an autoantigen.

Introduction

Humans infected with the Epstein–Barr virus (EBV) develop antibodies reactive with the nuclear proteins encoded by the viral genome (EBNAs). The immune

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response to the EBNA I and the EBNA 2 has been well characterized in its tim course and prognostic significance [1]. Antibodies to the EBNA 2 protein are th first to appear, reach a peak 4–12 months after infection and decline in tit thereafter; antibodies to the EBNA I appear several weeks or months after th anti-EBNA 2 and persist for the lifetime [1, 2].

The EBNA I has an unusual structure, as it contains in its central part a gly–al. repeat which constitutes one third of the molecule [3] and represents a dominan epitope in the anti-EBNA I immune response that follows EBV infection [2, 4] However, antibodies against other portions of the EBNA I molecule are present in immune sera. Milman *et al.* [5] detected in normal individuals antibodies reactive with a recombinant protein corresponding to the carboxyl terminal of the EBNA I that does not contain the gly–ala repeat. The fine specificity of these antibodies was investigated by means of synthetic peptides. Antibodies against the carboxy-terminus peptide 627–638 were mostly found in EBV-related diseases such as nasopharingeal carcinoma and Burkitt lymphoma, but were rare in normals [6]. Other sequences rich in glycine and arginine (peptides 331–344, 353–367 and 368–381) are recognized by serum antibodies in normals but especially in patients with rheumatoid arthritis and systemic lupus [7].

The N-terminal region of the EBNA I contains a sequence (35–58) which bears a strong homology to the C-terminal region of the nuclear protein SmD [8]. SmD, a core protein of the spliceosome, is one of the targets of antinuclear antibodies in systemic lupus (SLE). As autoantibodies to the Sm proteins are produced only in SLE, their presence is one of the serological criteria for diagnosis of SLE [9].

In a previous work [10], we detected antibodies to the C-terminal peptide of SmD in one third of SLE sera and showed that these antibodies react with the EBNA I 35–58 sequence and the whole EBNA I molecule as well. Immunizing mice with the EBNA I peptide in adjuvant, we were able to induce the production of anti-SmD antibodies. From these data we concluded that anti-SmD antibodies can be produced in the immune response to the EBNA I antigen by a process of molecular mimicry.

In the present report, we analyse the frequency of antibodies to the EBNA I 35–58 sequence in EBV-related disorders and in autoimmune diseases. Antibodies to the EBNA I 35–58 are present in autoimmune diseases and in EBV-related disorders as well. However, antibodies specific for this EBNA I sequence, affinity-purified from either autoimmune sera or from normal individuals, show a different pattern of reactivity: only antibodies from lupus sera react with both the EBNA I and the SmD peptide. The ability to produce anti-viral antibodies cross-reactive with a nuclear antigen seems therefore to be limited to SLE.

Materials and methods

Sera

Sera of patients affected by autoimmune disorders were obtained from persons attending the Clinical Immunology Unit, University of Pisa. Sera of patients affected by Burkitt lymphoma and nasopharingeal carcinoma were collected by Dr G. Klein, Tumour Biology, Karolinska Institut, Stockholm.

Sera of patients affected by infectious mononucleosis were a kind gift of Dr C. Garzelli, Dipartimento di Biomedicina, University of Pisa.

Peptide synthesis

Synthetic peptides were obtained by solid phase synthesis using F-moc protected aminoacids according to the method of Merrified, as modified by Atherton *et al.* [11]. The peptides were purified by gel filtration on a Sephadex G-25 column. The gly-ala repeat (EBNA GA: GAGGGAGGAGAGGGAGGAGA) sequence was synthesized as a linear peptide. The SmD 95–119 (AGRGRGRGRGRGRGRGR GRGGPRR) and the EBNA I 35–58 (GGDNHGRGRGRGRGRGGGRPGAPG) peptides were synthesized as MAPs. In accordance with Tam [12], a lysine scaffold with two terminal lysines was constructed and on the four amino groups of the two terminal lysines four identical linear sequences corresponding to SmD 95–119 or EBNA I 35–58 were synthesized.

A MAP bearing the tetanus toxoid sequence 947–967 (FNNFTVSFWLRVP KVSASHLE) [13] was used as a control for direct binding and inhibition assays.

Detection of anti-peptide antibodies

Peptides were used at a concentration of 10 μg/ml to coat polystyrene plates (Nunc, Denmark). After blocking for one hour with 3% BSA in PBS, sera diluted 1/500 in diluting buffer (1% BSA 0.05% Tween in PBS) were added to the plates and incubated for 4 hours at room temperature. The plates were washed once with 1% Tween in PBS and twice with PBS. An alkaline phosphatase-conjugated $(Fab)_2$ fragment of goat anti-human IgG (Sigma Chemical Co., St Louis, MO, USA) in diluting buffer was then added and incubated overnight at 4°C. After washings, the bound enzymic activity was measured with *p*-nitrophenyl-phosphate.

Ten sera that did not contain antibodies to the EBV capsid antigen (VCA-negative, collected by Dr Klein) were inserted in each test. Sera were considered positive when their optical density was higher than the mean plus two standard deviations of the VCA-negative controls.

For the competitive assays, 5% dry non-fat milk in PBS was used in the blocking step and PBS 2.5% milk, 0.05% Tween was used as diluting buffer for the sera and labeled antibodies. The amount of serum that gave 50% of the maximum binding was pre-incubated with different amounts of synthetic peptides or buffer for one hour at 37°C and then transferred to peptide-coated plates. The assay was then carried out as the direct binding assay.

Isolation of anti-peptide antibodies

EBNA I 35–58 was coupled to CnBr-activated Sepharose following the manufacturers' instructions. The sera were absorbed on peptide-coupled Sepharose; the bound antibodies were eluted by 0.1 M glycine pH 2.8 and dialysed against PBS.

The immunoglobulin content of eluates and flow-throughs from peptide columns was measured by ELISA. Briefly, polystyrene plates were coated with anti-human

IgG antibodies (SIGMA) diluted 1/1000, blocked with BSA and incubated with eluates and flow-throughs at various dilutions. The bound IgG antibodies were detected by addition of alkaline phosphatase-conjugated goat anti-human IgG.

Anti-SmD activity

Recombinant SmD molecule, a kind gift of Dr Pruuijn and Dr Van Venrooji, is expressed in the Studier expression system [14] as a fusion protein with 40 added amino acids, encoded by vector and linker. After the induction of expression, bacteria were lysed by repeated freezing–thawing and sonication. The lysate was centrifuged and the pellet containing the SmD protein was dissolved in 8 M urea, 25 mM Tris pH 7.5.

The bacterial products were separated on a 15% acrylamide gel under reducing conditions and transferred to nitrocellulose. The filters were saturated by one hour incubation in 0.05 M Tris 0.15 M NaCl 5% dry non-fat milk. The same buffer was used for antibody dilutions and washings. Purified antibodies at 30 µg/ml were incubated on filters for 4 hours at room temperature. After repeated washings, alkaline phosphatase-conjugated goat anti-human IgG (SIGMA) was added and the filters were incubated overnight at 4°C. The immunoactive bands were visualized using 5-bromo-4-chloro-indoxyl-phosphate and nitroblue tetrazolium as substrate [15].

Isolation of anti-SmD antibodies

The nitrocellulose strip containing the recombinant SmD was excised from blotted bacteria lysate. The strip was saturated in 0.05 M Tris 0.15 M NaCl 5% BSA and incubated with SLE sera diluted 1:20 in 0.05 M Tris 0.15 M NaCl for one hour at room temperature. The strip was washed five times in the same buffer, then the bound antibodies were eluted by 0.1 M glycine pH 2.8 and immediately neutralized by Tris.

Anti-EBNA I activity

The DG75 EBV-negative Burkitt lymphoma line and the EBNA I-transfected DG75 EBNA I were described elsewhere [16, 17].

Total cell extracts were prepared by sonication of 10^7 cells in 1 ml of sample buffer (Tris buffer containing 5% SDS and 5% beta-mercaptoethanol); they were boiled for 5 min, run on a 7.5% acrylamide gel (10^6/lane) and blotted to nitrocellulose. The filters were probed with affinity purified anti-SmD antibodies and with reference anti-EBNA I antibodies (affinity purified human antibodies specific for the EBNA I gly–ala repeat) [4, 17].

Results

Frequency of antibodies to EBNA I-derived peptides

Synthetic peptides from EBNA I (EBNA GA and EBNA I 35–58) and SmD (SmD 95–119) were used as antigens on the solid phase in an ELISA assay. Serum IgG

BSA and incubated
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IGMA) was added an
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5 M NaCl 5% BSA and
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cells in 1 ml of sample
ptoethanol); they were
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d anti-SmD antibodies
fied human antibodies

eptides

5–58) and SmD (SmD
ISA assay. Serum IgG

…able 1. *Antibodies specific for the EBNA I peptides GA and 35–58 and for the SmD 95–119 peptide in normals, EBV-related disorders and autoimmune diseases*

	EBNA GA	EBNA I 35–58	SmD 95–119
…mals	15/20 (75%)	6/20 (30%)	1/20 (5%)
…kitt lymphoma	11/16 (69%)	2/16 (12%)	2/16 (12%)
…opharingeal carcinoma	17/19 (89%)	7/19 (37%)	0/19 (0%)
…ctious mononucleosis	6/23 (26%)	5/23 (22%)	4/23 (17%)
…umatoid arthritis	23/36 (64%)	9/36 (25%)	1/36 (3%)
…stemic lupus	34/40 (85%)	15/40 (38%)	13/40 (32%)
…gren' syndrome	8/12 (67%)	4/12 (33%)	0/12 (0%)

…era from normals, EBV-related disorders and autoimmune diseases were incubated on peptide-…ed plates. Bound IgG antibodies were measured by addition of alkaline phosphatase-labelled …-IgG antibodies.
…era were considered positive when their optical density was higher than the mean plus two standard …ations of 10 VCA-negative controls (see Materials and Methods).

…ntibodies specific for these peptides were measured in EBV-associated diseases as …ell as in autoimmune disorders. The results, summarized in Table 1, confirm that …ost sera react with the EBNA GA, while EBNA I 35–58 is a minor epitope in …erms of frequency. However, antibodies with this specificity are produced in a …ariable percentage of subjects with EBV-associated diseases or autoimmune …isorders. Anti-EBNA I 35–58 antibodies are in fact present in 30% of normals, 12% Burkitt lymphoma, 37% nasopharingeal carcinoma and 22% infectious mononucleosis. Among autoimmune sera, anti-EBNA I 35–58 antibodies are detected in 25% rheumatoid arthritis, 33% Sjögren' syndrome and 38% systemic lupus sera.

On the contrary, antibodies against the SmD 95–119 peptide are present in 32% of lupus sera, 17% of infectious mononucleosis and 12% of Burkitt lymphoma sera.

Anti-Sm antibodies were measured by counterimmuno-electrophoresis, using rabbit thymus extract as antigen, in lupus, rheumatoid arthritis, infectious mononucleosis and normals. Only 6/40 lupus sera were positive; these sera contained antibodies to the SmD 95–119 peptide by ELISA.

All the sera, from any disease group, reactive with the SmD peptide bind the EBNA I 35–58 as well; several sera react with the EBNA I 35–58 but not with the SmD peptide. These results suggest the possibility that sera contain different subsets of anti-EBNA I 35–58 antibodies and only few of these cross-react with SmD.

Specificity of antibodies to EBNA I 35–58

To better characterize the relationship between anti-SmD and anti-EBNA I reactivities, serum antibodies specific for the EBNA I 35–58 peptide were affinity-purified over a peptide column. Anti-EBNA I 35–58 antibodies were purified from sera with a high titer of anti-peptide antibodies: two rheumatoid arthritis sera, three

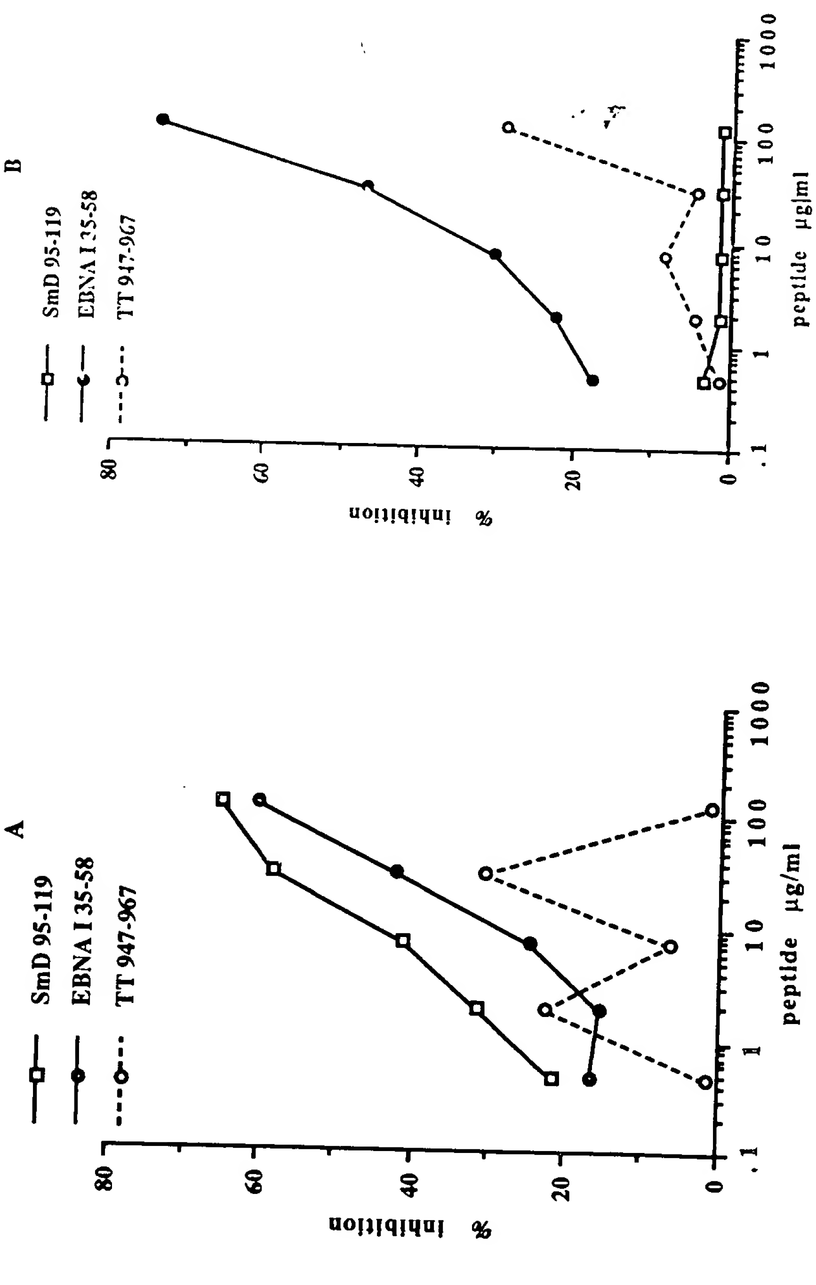
A
SmD 95-119
EBNA I 35-58
TT 947-967
% inhibition
80
60
40
20
0
.1
1
10
100
1000
peptide µg/ml
B
SmD 95-119
EBNA I 35-58
TT 947-967
% inhibition
peptide µg/ml

Figure 1. A,B.

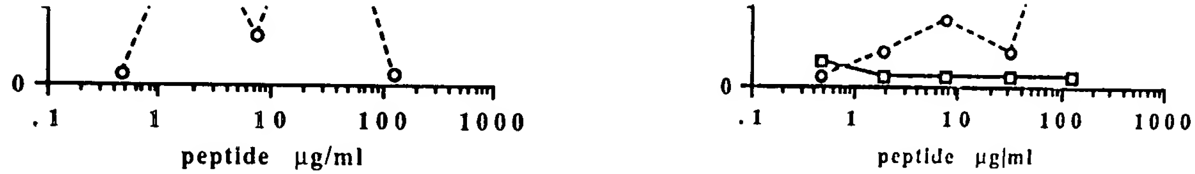

Figure 1. A,B.

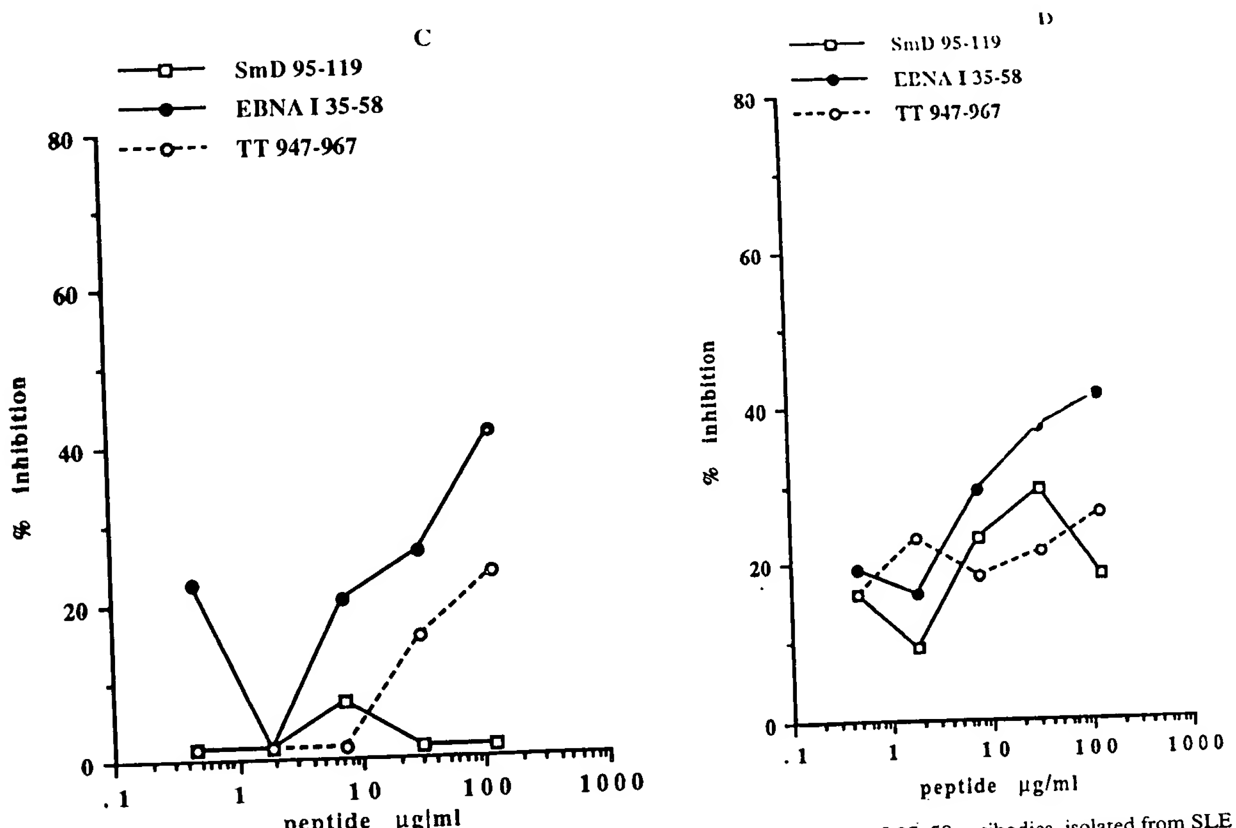

Figure 1. Inhibition of the binding of anti-peptide antibodies to EBNA I 35–58. Anti-EBNA I 35–58 antibodies, isolated from SLE (A), rheumatoid arthritis (B), Sjögren' syndrome (C) and normal sera (D), were preincubated with the different peptides at various concentrations and then transferred to EBNA I 35–58-coated plates. Bound IgG antibodies were detected by alkaline phosphatase-labeled anti-human IgG antibodies.

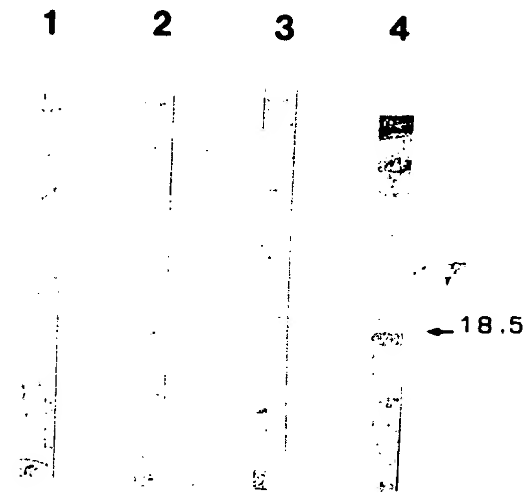

Figure 2. Binding of affinity purified anti-EBNA I 35–58 antibodies to recombinant SmD protein. The lysate from bacteria transfected with SmD was run on a 15% SDS gel under reducing conditions and transferred to nitrocellulose. The filters were probed with sera eluates from an EBNA I 35–58 column. Eluates from one rheumatoid arthritis (1), one SLE (2) and one normal serum (3) were tested. Anti-peptide antibodies from lupus serum detect a band of 18 kD, that corresponds to the molecular weight of the recombinant SmD molecule, as confirmed by the control monoclonal anti-SmD antibody shown in lane 4.

lupus, two Sjögren' syndrome and two normal sera. Antibodies eluted from the EBNA I 35–58 column specifically bound the solid phase EBNA I and SmD peptides; the ability to bind both peptides was markedly reduced in flow-throughs from the column (data not shown). The specificity of anti-EBNA 35–58 antibodies was evaluated by means of an inhibition assay. Binding of lupus anti-peptide antibodies to the EBNA I peptide on the solid phase was inhibited by both the liquid-phase peptide and the SmD peptide (Figure 1A). On the contrary, no inhibition was obtained with the SmD peptide in rheumatoid arthritis (Figure 1B). Anti-peptide antibodies from Sjögren' syndrome and normal sera (Figures 1C and D) had a lower affinity for the viral peptide, since only 40% inhibition was achieved with 100 μg/ml of peptide. As in rheumatoid arthritis, inhibition exerted by SmD was similar to that obtained with the control peptide.

These results indicate that only anti-EBNA I 35–58 antibodies from lupus sera cross-react with SmD.

Reactivity of antibodies to EBNA I 35–58 with recombinant SmD

To further analyse the reactivity of anti-EBNA I 35–58 antibodies, the eluates from the peptide column were tested on blotted recombinant SmD. As shown in Figure 2, only anti-peptide antibodies from lupus sera recognize the recombinant SmD. Thus, only anti-peptide antibodies from SLE sera cross-react with the SmD peptide and the whole SmD protein.

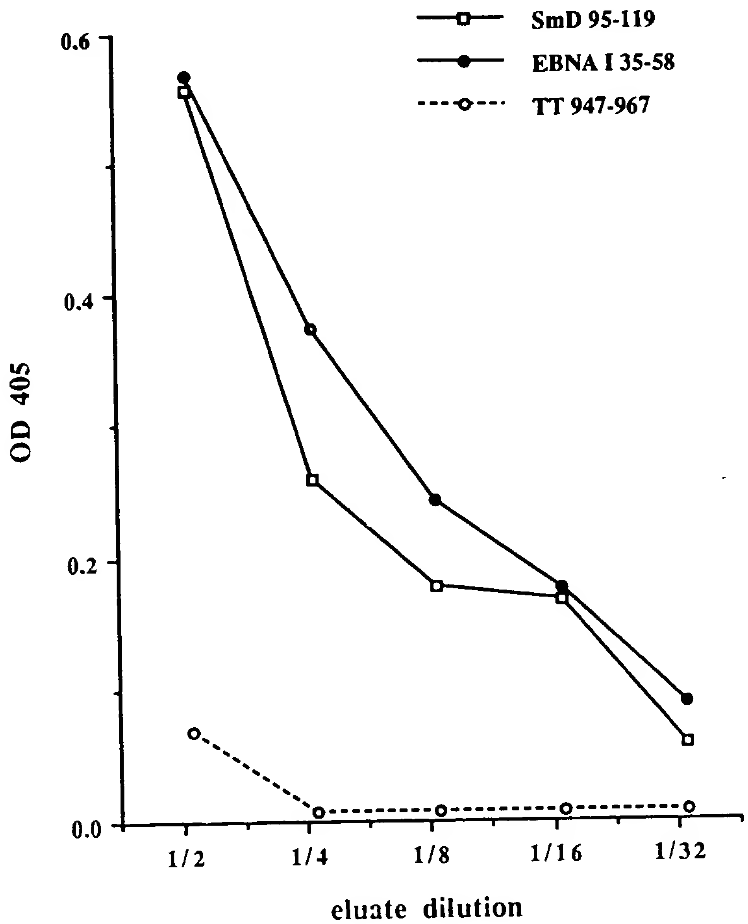

Figure 3. Binding of anti-SmD antibodies to SmD and EBNA I peptides. Anti-SmD antibodies were purified by acid elution from blotted recombinant SmD. The eluted antibodies were incubated at various dilutions on peptide-coated plates. Bound IgG antibodies were detected by alkaline phosphatase-labeled anti-human IgG antibodies.

Reactivity of anti-SmD antibodies with EBNA I

Anti-SmD antibodies were purified by acid elution from blotted recombinant SmD. The eluted antibodies bind the SmD and EBNA I peptides, as shown in Figure 3. Further evidence for the cross-reactivity of anti-SmD antibodies with EBNA I was obtained from the analysis of EBNA I-transfected lines. As shown in Figure 4, anti-SmD antibodies and reference anti-EBNA I antibodies detect a band of 76 kD expressed only in EBNA I-transfected lines.

Discussion

The data presented in this paper describe a new complex epitope in the N-terminal portion of the viral protein EBNA I, contained in a region of the molecule highly

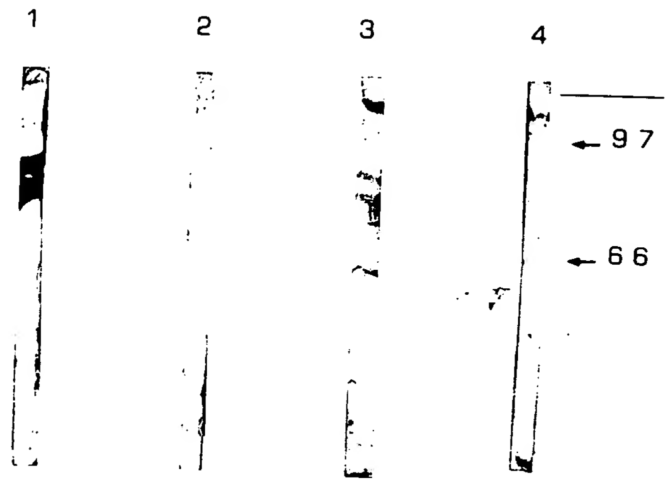

Figure 4. Binding of anti-SmD antibodies to EBNA I. Total cell lysates from EBNA I-expressing lines (filters 1, 3) and EBNA I-negative lines (filters 2, 4) were separated on a 7.5% acrylamide gel under reducing conditions and transferred to nitrocellulose. Nitrocellulose stirps were probed with reference anti-EBNA I antibodies (1, 2) or affinity-purified anti-SmD antibodies (2, 4). Anti-SmD and reference anti-EBNA I antibodies detect a 76 kD band present only in EBNA I-expressing lines.

homologous to the nuclear antigen SmD. Antibodies specific for this sequence are produced in normals as well as in subjects affected by EBV-related disorders or autoimmune diseases. Despite the structural similarities, antibodies against the SmD peptide have a very different distribution: they are in fact detectable in 32% of SLE sera, 17% of infectious mononucleosis and in a very low frequency in the other disorders.

The fine specificity of anti-EBNA I 35–58 antibodies is, however, different in the various disorders: only anti-peptide antibodies from lupus sera recognize the viral and the SmD peptide with a similar affinity. The ability to produce antibodies to the EBNA I that cross-react with a nuclear antigen seems therefore to be limited to SLE. These results support our previous findings, indicating again that in the immune response to the EBNA I antigen anti-SmD autoantibodies can be generated by molecular mimicry.

Antibodies to the EBNA I 35–58 are present in 12 to 37% of sera and their frequency is not strikingly different in autoimmune or EBV-related disorders. Thus, the ability to mount an immune response to this epitope is not a rare event nor is it confined to a single disease. The production of anti-EBNA I 35–58 antibodies of different specificity (some are inhibited by SmD, some are not) indicates that this sequence contains more than one epitope. Shorter overlapping peptides should be synthesized to precisely map the epitopes recognized by serum antibodies in the different disorders. However, it is conceivable that SLE antibodies recognize mainly the gly–arg stretch shared with SmD, while RA antibodies react with other sequences contained in the 24 amino-acid peptide. Epitope mapping by means of synthetic peptides has obvious limitations: in fact, only linear epitopes can be mapped. It is likely that most epitopes on a protein are conformational (e.g. formed by the three-dimensional folding of a polypeptide chain) and many linear epitopes

on the contrary, hidden inside the protein and exposed only by its unfolding [illegible]. However, the EBNA I 35–58 sequence is strongly charged because of its unusually high arginine content and should therefore be exposed on the surface of the native molecule.

Antibodies to the 35–58 epitope are produced early after infection, since 22% of infectious mononucleosis sera contain anti-EBNA I 35–58 antibodies. The production of anti-EBNA GA antibodies follows a similar time course: in fact we detect them in 26% of infectious mononucleosis sera. The late appearance of anti-EBNA antibodies reported in the literature is based on detection by immunofluorescence [illegible]; the ELISA assay, as already observed by Rhodes *et al.* [2], allows earlier detection. The frequency of anti-EBNA GA antibodies we detect is, as previously reported [2, 7], very high in normals and in the various disease groups, confirming that the gly–ala repeat is the dominant epitope in the human immune response to EBV.

The vast majority of sera containing antibodies to the 35–58 sequence contains antibodies to the gly–ala as well, but no correlation was found between the amounts of the two antibodies. The frequency and amounts of anti-EBNA GA antibodies are quite similar in normals and in EBV-related or autoimmune disorders. A marked difference between normals and rheumatoid arthritis has been reported [19], but these findings were not confirmed by later studies in which only small differences in antibody titers were detected [7, 20]. These antibodies, however, could play an important role in the pathogenesis of rheumatoid arthritis. It has been shown that affinity-purified anti-EBNA GA antibodies cross-react with collagen and keratin [21, 22]. Both proteins contain stretches of glycine and alanine that resemble the repeat sequences of EBNA I and anti-collagen and anti-keratin antibodies could be produced as part of the anti-EBNA I immune response. Although the presence of anti-collagen antibodies is not a constant finding in rheumatoid arthritis nor is it clearly related to the early phases of the disease or to the relapses [23], their pathogenic role is it clearly established in animal models of the disease [24, 25].

Thus, molecular mimicry with EBNA 1 could play a role in the induction of two types of autoantibodies, anti-collagen and anti-SmD, which have a quite different disease association. Anti-SmD antibodies are in fact present only in SLE patients, while anti-collagen antibodies are produced in rheumatoid arthritis, ankylosing spondilytis, systemic sclerosis and SLE. The HLA gene set and the T-cell receptor repertoire might be responsible for the production of anti-EBNA I antibodies cross-reactive with a particular autoantigen. Once triggered, the production of anti-SmD or anti-collagen antibodies is persistent in SLE and RA patients, with possible fluctuations linked or not to disease activity. In normal subjects, on the contrary, the production of autoantibodies induced by a viral infection is rapidly down-regulated. Rhodes *et al.* [26] found in acute infectious mononucleosis sera anti-EBNA I IgM antibodies reactive with both the EBNA GA and 10 cellular proteins; IgG antibodies produced during late convalescence do not cross-react with autoantigens. Similarly, we detected in 4/23 infectious mononucleosis sera anti-SmD 95-119 antibodies. The production of these antibodies is probably a transient phenomenon. In fact, even if we could not test sequential samples from these subjects, anti-SmD antibodies are not detectable in normals or in rheumatoid arthritis or Sjögren' syndrome patients. The control mechanism that suppresses

production of autoantibodies acts, in the case of the anti-EBNA I antibodies described by Rhodes *et al.*, at the level of the isotype switch from IgM to IgG. The anti-SmD antibodies transiently produced in infectious mononucleosis are, on the contrary, IgG. This finding underlines the complexity of the control mechanisms that regulate the immune response to different epitopes of a viral protein and the production of anti-self antibodies.

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